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Programme & Abstracts




PSE Congress

Plants for Human Health in the Post-Genome Era

26.8.- 29.8.2007, Helsinki

VTT SYMPOSIUM 249

Keywords: Bioactivity, genetic engineering, health effects, plant biotechnology, plant secondary metabolites, production, recombinant proteins

PSE Congress

Plants for Human Health in the Post-Genome Era

August 26–29, 2007

Rantapuisto Congress Centre

Helsinki, Finland

Edited by

Annemari Kuokka-Ihalainen
Kirsi-Marja Oksman-Caldentey
Heiko Rischer
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Preface

Dear Colleagues,

On behalf of the Phytochemical Society of Europe (PSE) and the organizing committee I have the pleasure to welcome plant scientists from all over the world to Helsinki, Finland to attend this most exciting congress entitled *"Plants for Human Health in the Post-Genome Era"*. This event has attracted close to 200 participants from 33 countries. It is organized by VTT Technical Research Centre of Finland, a leading non-profit research institute in Northern Europe. The congress takes place at Rantapuisto Congress Centre in a beautiful location by the Baltic Sea about 10 km from Helsinki city centre.

The aim of this congress is to bring together the scientists from academia and industry to share the recent developments in plant science and to discuss future prospects. When putting the program together the idea was to give the participants an overview on plant-derived compounds in respect to human health, the progress in rapidly developing research of recombinant proteins and secondary metabolites, and finally to get an industrial view on biotechnological applications using large-scale production of plant cells. The congress brings together 30 invited world leading scientists from Europe, USA, Canada, Japan and South Korea. In addition to the invited lectures four short presentations were selected by the scientific committee from submitted abstracts. Furthermore, there will be 65 posters with high scientific quality. All posters will be available during the whole time of the congress which is anticipated to stimulate interesting and inspiring discussions.

I would like to thank warmly the local organizing committee members, the international scientific committee and the sponsors. Without the financial support of the industrial and organizational sponsors this congress would not have been possible. Special thanks go to Ms Katri Luomanpää (Congreszone) and Ms Annemari Kuokka-Ihalainen (VTT) for their excellent organizational and secretarial help.

My hope is that during this meeting everybody will have an excellent possibility to meet colleagues, to develop new friendships and to establish fruitful collaborations. I wish you all a very successful congress and a pleasant stay in Helsinki.

Kirsi-Marja Oksman-Caldentey
Chair of the Organizing Committee

Organising committee

Kirsi-Marja Oksman-Caldentey (Chair)
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Anneli Ritala-Nurmi (Espoo, Finland)
Robert Verpoorte (Leiden, The Netherlands)

Contents

Programme

Sunday 26.8.2007

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14.00–14.30		Opening remarks ➢ Kirsi-Marja Oksman-Caldentey *Chair of the Organizing Committee* ➢ Vasillios Roussis *Chairman of PSE* ➢ Hans Söderlund *Welcome address from VTT*
14.30–15.30	O1	Opening plenary lecture: Indra Vasil (University of Florida, USA) *Fifty Years of Plant Biotechnology: 1957–2007*
15.30–16.00		Coffee break
16.00–16.45	O2	Keynote lecture: Dirk Inzé (VIB, Belgium & EPSO) *Plant Science for a Sustainable Europe*
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		**********
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		**********
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Tuesday 28.8.2007 **Session 5: Plants as Factories for Pharmaceuticals**
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Chair: Wilhelm Gruissem and Richard Dixon

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		**********
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Part 2: Recombinant proteins

Chair: Anneli Ritala and Rainer Fischer

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12.00–13.00		Lunch

Chair: Maurice Moloney

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Session 7: Large-Scale Production for industrial applications

Chair: Heiko Rischer and Venkatesh Srinivasan

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16.00–16.30	O29	Jean-Paul Ducos (Nestlé R&D Centre, France) *Bioreactors for Plant Science: Production of Ingredients and/or Somatic Embryos of Improved Species?*

Session 8: Future outlook

Chair: Kirsi-Marja Oksman-Caldentey

16.30–17.00	O30	Teemu Teeri (University of Helsinki, Finland) *Where do we go from here: The Next Decade of Plant Biotechnology*
17.00–17.15		Closing remarks (*Kirsi-Marja Oksman-Caldentey*, VTT, Finland)
		**********
19.00–21.00		Dinner *(optional)*

ABSTRACTS OF
ORAL PRESENTATIONS

INVITED LECTURES
O1–O30

Fifty years of plant biotechnology: 1957–2007

I. K. Vasil

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Genetic transformation of plant cells, and regeneration of plants from cultured cells, together have provided the experimental basis for the development of plant biotechnology during the past fifty years, leading to the production and commercialization of many transgenic crops. These crops with novel and useful traits are beginning to make important contributions to food security, economic development, and conservation of the environment and biodiversity. Plant biotechnology is founded on the concept of cellular totipotency, the theoretical and conceptual framework for which was provided by the Cell Theory formulated by Schleiden and Schwann in 1838/1839. The subsequent discovery of plant growth substances and their role in morphogenesis, the demonstration of totipotency, the invention of methods for the transformation of plant cells, and the sequencing of plant genomes have all been major turning points in the evolution of plant biotechnology. This paper traces this fascinating history, and celebrates the pioneering and trend-setting contributions of the men and women who made it all possible.

Plant science for a sustainable Europe

D. Inzé

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Our world economy is not sustainable and alarming signs such as global warming urges for finding efficient alternative, non-polluting, production systems and renewable energy resources. Plants have the capacity of converting the excess solar energy that continuously hits the earth into numerous organic substances many of which have already an industrial use (e.g. starch, oils, rubber, pharmaceuticals, wood). Plant science has spectacularly advanced in recent years and much of the current and future knowledge will become directly applicable to improve crops already in use and to select novel crops for fueling our economy. Plants, for example, can be engineered to produce effectively novel starches, modified oils, biodegradable plastics, and high-value materials. However, the effective use of plant derived products will not only dependent on our ability to modify plant metabolic pathways but will also require the development of effective down stream processing methods of plant materials. A “green chemistry” needs to be developed to use, as effective as possible, biomolecules produced by plants, including very complex ones such as lignin. We need to develop a “no-waste-no-pollution” economy in which all plant derived materials are recycled. I strongly believe that plant scientists have to work much more closely with pharmacists, organic chemists, process engineers and microbiologists. We need to enhance the dialogue between all these disciplines in order to find more effective uses of plants and plant scientists can select or engineer plants with are much better adapted to the needs of the industry.

Opportunities of plant biotechnology for developing countries

W. Gruissem, S. Poletti, C. Sautter, M. Stupak, H. Vanderschuren and P. Zhang

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Plant biotechnology can make important contributions to food security and nutritional improvement. The development of "Golden Rice" by Prof. Ingo Potrykus is a milestone in the application of gene technology to deliver both increased nutritional qualities and health improvement to broad segments of the human population. However, mineral and protein nutrient deficiencies as well as food security remain as some of the most important challenges for developing countries (and surprisingly, for several European countries as well). Our current projects address these issues in two major staple crops, cassava and rice. Although not well known in Europe, the tropical root crop cassava (*Manihot esculenta* Crantz) is a major source of food for approximately 800 million people worldwide. In sub-Saharan Africa alone, more than 200 million people rely on cassava as their major source of dietary energy. Cassava production is threatened by viruses, however, and the nutritional quality of the cassava root is not sufficient to meet all dietary needs. We have developed transgenic cassava with resistance to African Cassava Mosaic Virus infections, improved protein levels, and enhanced leaf life and drought resistance. The robustness of the new cassava traits is under evaluation in field tests in China and South-America, and field tests are in preparation in Africa.

Rice is the staple food for half of the world population. It provides some 20% of the per capita energy and 13% of the protein for human consumption worldwide. In many developing countries, the dietary contributions of rice are substantially greater, 29.3% energy and 29.1% protein, respectively. The current top six popular mega rice varieties (in terms of popularity and acreage), including Chinese hybrid rice, have an incomplete amino acid profile and contain limited amounts of essential micronutrients. We have constructed rice lines with improved Fe content using genes for functions in absorption, translocation and accumulation in the plant, as well as bioavailability in the human intestine. In collaboration with nutritionists in the U.S. and Switzerland, these lines have moved into the evaluation phase. We will review current benefits of biotechnology-assisted plant improvement and illustrate the controversy that has developed around gene technology in plants, especially in European countries. As the world population continues to grow, mostly in under-developed countries, and gentrification becomes a concern of many developed countries, this controversy must be considered in the context of agricultural production that in the future will have to address a secure and healthy food supply.

Needs for new plant-derived pharmaceuticals – industrial view in drug research and development

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With the completion of sequencing the human genome, we are entering the post-genomic era that concentrates on harvesting the fruits hidden in the genomic text. The advent of genomics and the molecular biology revolution has permitted both the definition of new targets and the characterization of the genetic basis of disease states. The introduction of various powerful new technologies have also greatly accelerated the pace of new drug discovery. All these progresses create immense opportunity for plant-derived pharmaceuticals, which once served as the source of all medicinal agents in the ancient time and also provided many important therapeutic entities in contemporary drug research and development.

Discovering novel targets not already hit by existing drugs is the prerequisite for the success to find innovative new drugs. In target directed drug discovery, candidates act on specific targets offer the advantage that the mechanism of drug action can be well understood and accurately monitored in preclinical research and clinical trials. Since the native use of medicinal plants in traditional medicine constitutes a kind of pre-existing clinical testing, investigations on their mode of action should increase the success probability to find novel targets. In another aspect, the specific folk-medicinal uses provide a very valuable short cut to biologically active compounds with particularly added advantages to related diseases.

Following the maturation of combinatorial chemistry and compound library development for lead finding, it has been recognized that biological relevance and chemical diversity are more important than the library size. As chemical defenses against insect, microorganisms and other predators in their evolution, higher plants are remarkable in their ability to produce a vast array of diverse metabolites varying in chemical complexity and biological activity. Therefore, high throughput isolation of plant metabolites can effectively supplement chemical compound libraries and combinatorial synthetic efforts.

To further increase chemical diversity for hit finding, biotechnological production of plant secondary metabolites can be enhanced by the treatment of undifferentiated cells with various elicitors. Advancement in bioconversion and combinatorial biosynthesis is promoting their application in lead optimization and drug development. Functional genomics approaches are accelerating plant gene discovery for biosynthetic pathways of plant metabolites and their regulation, which will also improve the production sustainability and efficiency of plant-derived pharmaceutical.

Genetic engineering of food crops for production of health-related compounds

A. Bovy, E. Schijlen, J. Schaart, H. Schouten, J. Beekwilder and R. de Vos

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Plants and their products are generally known for their high levels of antioxidants, which may contribute to the positive effects of dietary plant products for human health. Plants produce these compounds to cope with the biotic and abiotic stress environment they are growing in (e.g. UV-light, air and soil pollution, pathogens) as well as for their reproduction (e.g. pollination, pigmentation of flowers and fruits). Depending on plant species, variety and tissue, high levels of health-related antixoxidants, such as vitamin C and E, flavonoids, and/or carotenoids can be found.

Flavonoids comprise a large and diverse group of polyphenolic plant secondary metabolites. Flavonoids are widespread among the plant kingdom and form an integral part of the human diet. There is increasing evidence that dietary flavonoids are likely candidates for the observed beneficial effects of a diet rich in fruits and vegetables on the prevention of chronic diseases. Although some plants, such as onions (flavonols), blueberries (anthocyanins) and soybean (isoflavonoids) contain high levels of certain flavonoids, in other species the composition of these secondary metabolites is 'sub-optimal'. In addition to classical breeding, genetic engineering is an effective strategy to develop food crops with an optimal flavonoid composition. In our laboratory we investigated the possibilities of engineering the biosynthesis of flavonoids in several commonly consumed vegetables and fruits. For instance, in tomato we were able to greatly increase the level of existing flavonols by overexpressing genes encoding the rate-limiting enzyme or by introducing transcription factor genes. Several health-related flavonoids completely novel for tomato were obtained by directing the pathway towards new branches through the expression of genes from other plant species. In addition, specific branches in the flavonoid pathway could be blocked through RNA interference, leading to a reduction in specific flavonoids and an increased flux towards other flavonoid classes. Similar strategies are currently being employed to optimise the levels of anthocyanins in apple fruit and proanthocyanidins in strawberry.

These genetically engineered, nearly-identical plants, only differing in their levels of flavonoids are excellent material to study the potential health effects of dietary flavonoids present in a food matrix. In fact, in a recent study with mice it was shown that the consumption of tomatoes positively affected the expression of cardiovascular risk markers and that this beneficial effect was significantly enhanced with transgenic flavonoid-rich tomatoes.

In addition to genetic engineering of crop plants, we aim to modify and optimise the bioavailability and functionality of flavonoids in processed foods, through the expression of flavonoid modifying genes in micro-organisms and their subsequent use in bioconversion studies. The potential of this approach will be discussed.

Targets for nutritional enhancement in fruit: Pitfalls, shortcuts and progress

D. Stewart[1], G. J. MacDougall[1], R. M. Brennan[2], J. Graham[2] and I. Martinussen[3]

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Supporting for the beneficial health effects of fruit is accruing apace. A subdivision of fruit, the berries, are increasingly becoming the focus of studies regarding their proposed ability to prevent or ameliorate the problems of degenerative diseases.

With respect to berries there has, over the last decade, been a groundswell of reports attributing beneficial biological activity to the fruit phenolics. The predominant approach in these studies is that of well defined *in vitro* systems employing mammalian cell models systems, such as Hela, Caco2, HT29, Hep G2, etc. to study e.g. absorption, anticancer and metabolism effects. However, the direct translation of the benefits reported in these *in vitro* studies to *in vivo* results have lagged behind and are only now gathering pace. For example there are several intervention studies published highlighting or attributing their beneficial effects (albeit sometimes marginal) with regard to markers of colon and oesophageal cancer, cardiovascular disease, etc to the polyphenolic components in fruit. In addition, there are several major intervention trials either ongoing or planned and their focus is on fruit such as strawberry (cholesterol lowering), pomegranate (prostate cancer), blueberry (inflammation) and blackcurrant (CVD).

This positive evidence with respect to the efficacy of fruit in the diet as a potential strategy to prevent, or at least retard, chronic and/or degenerative disease is leading to enhanced nutritive value now becoming a major target for plant breeders. However the lack of clarity as to the actual target means that breeding is not straight forward. Due to the chemical diversity of fruit, and specifically in berries, newer screening approaches have been adopted; metabolomics – LC-MS^n, GC-ToF-MS^n NMR etc. We will discus how these approaches are being used in fruit breeding to study the inheritance of multiple silent phenotypes (chemotypes) in concert with map-based genetic approaches with a view to nutritional enhancement.

Colon, a forgotten site of human xenobiotic metabolism?

A.-M. Aura, I. Mattila, T. Seppänen-Laakso, M. Oresic and K.-M. Oksman-Caldentey

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Human colon contains 1.5 kg of microbiota, which actively takes part in the degradation and decomposition of the non-absorbable intake. This is common knowledge in the dietary fibre research. The concept of dietary fibre complex was proposed in 1984 including polysaccharides and ubiquitous phenolic compounds entrapped into the plant matrix. These phenolic compounds include flavonoids, phenolic acids, tannins, stilbenes and plant lignans, of which lignans are the most studied in terms of microbial metabolism in the colon. Furthermore, there is strong evidence on correlation between their intake, plasma concentration of microbial metabolites, enterodiol (END) and enterolactone (ENL), and reduced risk of chronic diseases.

Colonic microbiota changes by age, diet, intestinal diseases and medication causing intra-individual variation in the metabolite pool in addition to the inter-individual variation between subjects. To address these challenges, the developed batch *in vitro* colon model can be coupled with an advanced metabolomics and bioinformatics platform to provide data on the circulating metabolites. This work has recently been performed for flavan-3-ol stereoisomers, (+)-catechin and (-)-epicatechin. A good correlation has been found for dietary phenolic microbial metabolites between the *in vitro* colon model and corresponding metabolite profiles from human body fluids. In pharmaceutical research colon has been considered only as an excretion route for non-absorbable drug remnants. Only few companies include microbial metabolism in their drug development and authorities have not given guidelines in this respect. In the case of statins, a cholesterol lowering drug, Simvastatin causes alteration in the pro-inflammatory pathways and in high doses the risk of statin induced myopathy increases. Major portion of Simvastatin is excreted via faeces and thus its adverse effects may be connected with yet unkown colonic metabolites, which identification is attempted.

Phytoestrogens as natural anticancer prodrugs – proof of a novel concept

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It has been generally accepted that regular consumption of fresh fruits and vegetables is linked with a relatively low incidence of cancers (e.g. breast, cervix, and colon). A variety of polyphenol compounds have been identified that are considered to play a role in cancer prevention. Roughly, two models that may explain the cancer preventive properties of polyphenols are cited time and time again: the phytoestrogen model and the antioxidant model. However, neither model can fully explain why certain naturally occurring compounds can prevent the occurrence of cancer.

The current review is an effort to develop a consistent and unambiguous model that explains how some plant-derived compounds can prevent tumour development. The model is based on recent discoveries in the fields of genomics and drug-metabolism; notably, the discovery that *cyp1* genes are highly expressed in developing tumour cells but not in the surrounding tissue, and that a variety of plant-derived compounds are substrates for the CYP1 enzymes.

Our hypothesis is that some dietary compounds act as prodrugs, i.e. compounds with little or no biological effect as such, but become pharmaceutically effective when activated. More specifically, we state that the abovementioned prodrugs are only activated in *cyp1*-expressing cells – i.e. cells in the early stages of tumour development – to be converted into compounds which inhibit cell growth. Thus, the prodrugs selectively kill precancerous cells early in tumour development. Preliminary data that underpin our hypothesis will be presented. The review focuses on the identification of naturally-occurring prodrugs that are activated by the tumour-specific CYP1 enzymes and aims to assess their role in cancer prevention.

High-throughput screening for cancer drug targets and leads

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Abstract not available

Transcript profiling and jasmonates, a mighty couple for gene discovery in plant metabolism

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Plants are capable of producing an overwhelming variety of secondary metabolites, both in terms of complexity and quantity. Yet, when compared with the situation in micro-organisms this impressive metabolic machinery is hardly exploited. One of the main reasons is the limited molecular insight into plant secondary metabolism, which hampers the design of effective metabolic engineering strategies. To address these shortcomings we employ a functional genomics based technology platform that enables comprehensive investigations and large-scale gene discovery programs in plant metabolism. The platform relies primarily on the integration of genome-wide transcript profiling with metabolite profiling and is applicable to any plant species or system to map the biosynthesis of any metabolite.

The platform starts from the rationale (i) that transcripts corresponding to genes involved in a particular metabolic pathway will be 'enriched' in cells in which the particular metabolites are produced and (ii) that patterns of co-expression can reveal associations between metabolic pathway genes. The plant stress hormone jasmonate is one such compound that can be employed universally across the plant kingdom to induce 'enriched' biosynthesis conditions for metabolites of different classes. By profiling jasmonate elicited plant cells or tissues of amongst others *Arabidopsis thaliana, Artemisia annua, Bupleurum falcatum, Catharanthus roseus, Glycyrrhiza glabra, Maesa lanceolata, Medicago truncatula, Nicotiana tabacum, Panax ginseng, Taxus baccata* and *Veratrum californicum*, we now have built an extensive collection of thousands of genes potentially involved in diverse aspects of plant secondary metabolism.

Several experimental approaches are being used in the ongoing functional analysis of these genes. Principally, effects of gain- and loss-of-function are investigated; both in transient expression assays in protoplasts and in stable transformed plants or *in vitro* cultures; and both in the plants from which the genes are derived and in heterologous plant systems. This study has served to pinpoint some conserved principles in the jasmonate mediated activation of plant secondary metabolic pathways.

Plant metabolomics for small molecules

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Metabolite measurements provide a snapshot of the metabolic status. However, the measurement of metabolites is complicated by (i) the vast chemical complexity, which means that multiple analytic platform are needed, (ii) chemical instability, which means that checks are needed of the reliability of the extraction and analysis procedures, (iii) the rapid biological turnover or metabolites, which means that care is needed in tissue handling and quenching, and (iv) the need to resolve cellular and subcellular compartmentation. The technical problems and potential solutions will be discussed, using examples drawn from studies of plant central metabolism.

I will then discuss how metabolite measurements can be used to provide insights into the system properties and regulatory networks. Examples will include the use of exhaustive analyses of the metabolites in a set of pathways to define the thermodynamic characteristics of a defined metabolic network, the exhaustive measurement of metabolites in combination with experimental perturbations and flux determinations to identify regulatory sites, analysis of metabolites to provide information about the systems properties of a sector of metabolism, comparison of metabolite, enzyme activity and transcript profiles to gain information about the dynamic structure of cellular networks, and the use of metabolites profiling in combination with other ‘omics’ technologies and natural diversity to analyse complex physiological traits.

Directing gene expression to chloroplasts

H. Kim, H. J. Chung, S. R. Min, W. J. Jeong and J. R. Liu

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Plant growth and productivity are governed primarily by photosynthetic carbon metabolism (Calvin cycle). In higher plants, fructose-1,6-bisphosphatase (FBPase) and/or sedoheptulose-1,7-bisphosphatase (SBPase) are factors limiting carbon flow through the Calvin cycle. We transformed tobacco plant chloroplast genomes with the gene encoding FBP/SBPase from the photosynthetic cyanobacterium *Synechocystis* sp. strain PCC 6803. In this organism, the protein functions as both an FBPase and a SBPase. Compared to control plants, chloroplast-transformants overexpressing the recombinant gene exhibited up to 5-fold higher plastidic FBPase and SBPase activity. This change was accompanied by a significant increase in the rate of CO_2 assimilation. This study demonstrates for the first time that introduction of a gene into the chloroplast genome is more effective for manipulation of the Calvin cycle than chloroplast-targeted gene expression.

Genetic engineering of plant natural product pathways

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The impact of plant natural products (phytochemicals) on human health is increasingly recognized. Many of the compounds of current interest have been known and studied for many years, but it is only with the advent of genetic and genomic approaches that their biosynthesis has been understood at a level to permit their engineering in crop plants. Evidence of nutritional value for various classes of plant natural products will be reviewed, and specific examples provided of the application of molecular genetic approaches towards natural product pathway engineering.

1) Proanthocyanidins (PAs) are flavonoid oligomer antioxidants, commonly found in seed coats. The flavanol (-)-epicatechin is a component of many PAs, and contributes to flavor and astringency in tea and wine. The BANYULS gene of *Arabidopsis* encodes an anthocyanidin reductase (ANR), which converts the flower pigment cyanidin to (-)-epicatechin. Co-expression of ANR with flavonoid pathway transcription factors leads to production of epicatechin and PAs in tobacco, *Arabidopsis* and *Medicago* species, although there are significant species-specific differences. In particular, the PAP1 transcription factor that controls anthocyanin accumulation in *Arabidopsis* and some heterologous species such as tobacco fails to induce anthocyanin production when expressed in legumes. The goals of this research are to engineer health-promoting antioxidant PAs into dietary food crops, and also into forage legumes to reduce pasture bloat potential and improve ruminant nitrogen usage efficiency.

2) Soybean seed products are the major dietary source of isoflavone phytoestrogens. However, isoflavones can be engineered into species that naturally lack the pathway. The level of production depends on the activity of endogenous competing pathways, and can be increased by blocking these pathways. The introduced isoflavones accumulate as various sugar conjugates depending on the species transformed.

3) Enzymatic modification (eg. *O*- and *C*-glycosylation, prenylation, *O*-methylation) of isoflavones and other flavonoids can lead to a range of novel products with enhanced/modified bioactivity.

Metabolomics – back to basics

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Metabolomics as the latest of the -omics has got quite some attention in the past years. metabolomics has the very ambitious objective to identify and quantify all metabolites in an organism. Numerous reviews have been written in the meantime pointing out the various advantages of the possible analytical methods. Also the way to store metabolomic data has been discussed. But where do we stand now, do we have public databases with the metabolomic data, similar as to gene and protein sequence databases? Can we expect these in the near future? What are the hurdles and can we overcome these? Many questions, few answers. Which is maybe not so surprising after all. The dream of all natural products people in the past 50 years has been to develop reproducible analytical methods for the analysis of compounds in plants (or any organism). However, from the journals in the field of chromatography and analytical chemistry we know that instead of a dream we rather have a nightmare. When we wrote our books on chromatography of alkaloids some 25 years ago, we reviewed the analysis of tropane alkaloids: 69 references concerned the HPLC and 32 the GC analysis. Every year since then new studies showed new methods with the same aim. Apparently standardization failed, and we have to develop new methods all the time. Reason is improving quality of equipment, and chromatographic materials. But even a seemingly trivial aspect, the method of sample preparation, is not yet standardized in natural products analysis. Major reason is the great variety of physical properties of the analytes. Harvesting, storage, grinding, extraction, there are many steps that carry the risk of artifact formation or loss of compounds due to e.g. insolubility. Consequently everybody makes his own choices, based on the question that needs to be answered, i.e. a targeted approach is used. For a total analysis of all metabolites we must conclude that we still have no suitable standard method.

Biochemical genomics to study benzylisoquinoline alkaloid biosynthesis in plants

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Opium poppy remains the world's most important medicinal plant due to its unique ability to synthesize the narcotic analgesics morphine and codeine. More than 80 benzylisoquinoline alkaloids have been identified in opium poppy, most of which are intermediates of a complex multi-branched pathway. The demonstrated and potential applications of a wide array of biochemical, molecular, cellular, and genomic techniques have elevated opium poppy to the status of a model system to study plant alkaloid biosynthesis. Broad-scope, high-throughput genomics has recently emerged as a powerful strategy to complement more traditional approaches. We have established EST databases based on the attempted sequencing of >30,000 cDNAs from opium poppy and >46,000 cDNAs from eight related benzylisoquinoline alkaloid-producing species representing four plant families. These resources provide a unique opportunity for comparative genomics and have already resulted in the isolation of several novel genes including norcoclaurine synthase (NCS) and tetrahydroprotoberberine *cis*-*N*-methyltransferase, the first AdoMet-dependent *N*-methyltransferase known to produce a quaternary ammonium alkaloid. NCS catalyzes an asymmetric Pictet-Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde to yield (*S*)-norcoclaurine, the central intermediate in benzylisoquinoline alkaloid metabolism. This unique reaction has prompted collaborative efforts to elucidate the catalytic mechanism and three-dimensional structure of NCS. Our functional genomics platform includes a 23,000-element opium poppy-specific DNA microarray that has been used to investigate transcript profiles in elicitor-treated cell cultures and plants with different alkaloid content. Correlations between transcript and metabolite profiles have been based on metabolomics strategies using Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS) and nuclear magnetic resonance (NMR) spectroscopy. Three unique cell types – phloem sieve elements, companion cells and laticifers – have been shown to participate in alkaloid metabolism in opium poppy, implicating roles for developmental regulation and the intercellular transport of pathway intermediates and products. In addition to novel biosynthetic enzymes, we are also considering the participation of genes encoding regulatory and transport proteins. Viral-induced gene silencing (VIGS) is effective in opium poppy and is under development as a functional genomics tool to investigate the function of unknown genes.

Camptothecin biosynthetic system – pathway elucidation, gene discovery and self-resistance

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Camptothecin, a plant-originated alkaloid, exhibits an antitumor activity due to its inhibitory action to DNA topoisomerase I. At present, semisynthetic water-soluble camptothecin analogues, topotecan and irinotecan, are prescribed as clinical antitumor drugs throughout the world. Despite its quinoline structure, camptothecin belongs biogenetically to a family of modified monoterpenoid indole alkaloids. However, the information about genes and pathway after strictosidine is limited.

We have established a hairy root culture of *Ophiorrhiza pumila* (Rubiaceae) which produce a high level of camptothecin and anthraquinones. This hairy root culture is a feasible system not only for practical production of camptothecin but also for gene discovery and pathway elucidation. From this hairy root culture, we have established the non-differentiated cell-suspension culture that does not produce these secondary products. Thus, the comparison of these hairy root and cell suspension cultures is a desirable experimental system for research of molecular biology and biochemistry of camptothecin biosynthesis. We have conducted PCR-select cDNA subtraction for those two cultures to isolate cDNA fragments which are specifically expressed in camptothecin-producing tissues. In addition, the full-length cDNA clones have been sequenced from the both sides. Functional identification of those cDNAs that are presumed to be involved in biosynthesis of camptothecin is now undertaken by RNAi strategy in transformed roots and analysis of recombinant proteins.

Camptothecin exhibits eukaryotic topoisomerase I poisoning activity, resulting in cell death. Because of its toxicity to a house-keeping enzyme of cells, we addressed the question how the camptothecin-producing plant cells survive in the presence of camptothecin. We hypothesized that these plants might possess camptothecin-resistant type topoisomerase I. In fact, the recombinant *Op*TOPI from *O. pumila* expressed in yeast was resistant to camptothecin. Amino acid sequence analysis of *Op*TOP1 revealed that the highly conserved residue next to the catalytic site has been mutated. Coincidently, the identical mutation has been observed with the camptothecin-resistant human topoisomerase I. To confirm that the mutation is concomitant with and presumably caused by the presence of camptothecin, we compared the amino acid sequence of topoisomerase I from *O. japonica,* which is closely related to *O. pumila* but does not produce camptothecin. As expected, no mutation was confirmed in this non-producing species. Our findings suggest the possibility of adaptive co-evolution of topoisomerase I with camptothecin biosynthetic pathway in camptothecin-producing plants.

Novel acetogenic natural products: Structural elucidation online, synthesis and biosynthesis

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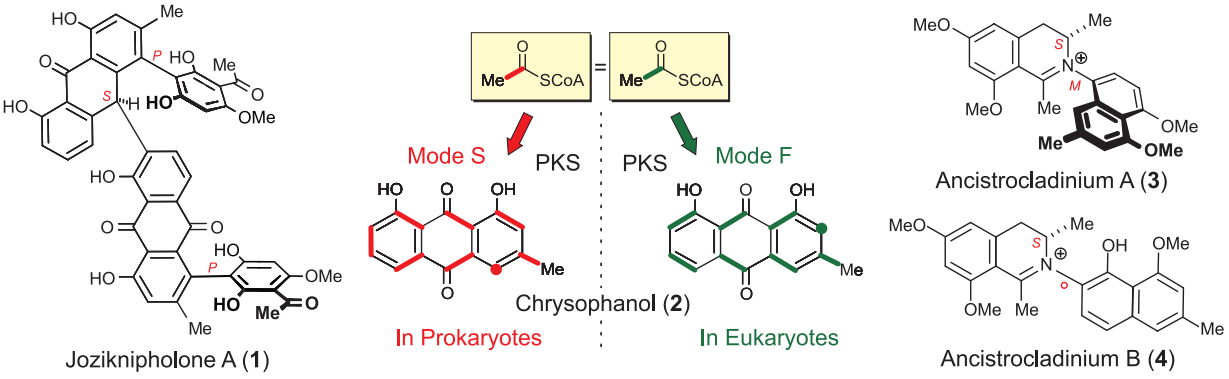
Nature provides a huge variety of structurally diverse natural products. Many of them originate from simple acetate-malonate units catalyzed by polyketide synthases (PKSs), often in combination with other enzymes. For the directed search for novel acetogenic metabolites right from crude extracts, we have composed the analytical triad LC-MS/MS-NMR-CD, in combination with quantum chemical CD calculations. The lecture describes the use of this methodology for the early recognition and online-structural elucidation of novel-type natural products, including their absolute configurations. Furthermore, their bioactivities, their total synthesis and, in particular, their biosynthetic origins are described.

Three such novel-type structures are joziknipholone A (**1**), an unprecedented dimeric phenylanthraquinone,[1] and ancistrocladinium A (**3**) and B (**4**), the first *N,C*-coupled naphthyldihydroisoquinoline alkaloids.[2]



Most remarkable is the biosynthesis of chrysophanol (**2**).[3] This anthraquinone portion of joziknipholone A and of its monomeric half, knipholone,[4] originates from at least two different folding modes, S and F, depending on the producing organism, while the naphthylisoquinoline alkaloids are the first acetogenic (and not aminoacid derived) isoquinoline alkaloids.

1. G. Bringmann *et al.*; The absolute axial configurations of knipholone and knipholone anthrone by TDDFT and DFT/MRCI CD calculations: a revision. *Tetrahedron*, submitted.
2. G. Bringmann *et al.*; Ancistrocladinium A and B, the first *N,C*-coupled naphthyl-dihydroisoquinoline alkaloids, from a Congolese *Ancistrocladus* Species. *J. Org. Chem.* 2006, *71*, 9348–9356.
3. G. Bringmann *et al.*; Different polyketide folding modes converge to an identical molecular architecture. *Nature Chem. Biol.* 2006, *2*, 429–433.
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Transporters for plant secondary metabolites

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Higher plants produce a vast number of secondary metabolites, which are mostly low molecular weight organic compounds but some are polymerized to form polymers, like condensed tannins. These natural compounds are classified according to their structures and biosynthetic routes into several groups, such as alkaloids, terpenoids, phenolic compounds, and further many compounds having combined structures of those groups. They are often accumulated in particular sink organs, and some of them are translocated from source cells via long distance transport.

The membrane transport of plant secondary metabolites has become an active research area. Genome sequencing projects and expressed sequence tag (EST) databases have revealed that many genes coding for transporters and channels exist in plant genome. Studies on phenotype analyses of many mutants by various criteria have identified some transporter molecules responsible for the membrane transport of plant secondary metabolites. Characterizations of such transporters on physiological and biochemical basis have clarified that the membrane transport for secondary metabolite is fairly specific and highly regulated. Not only genes that are involved in the biosynthesis of secondary metabolites but also genes relevant to their transport are important for high accumulation of those metabolites.

Membrane transport of solutes is roughly divided into two groups, primary transport using the energy of ATP hydrolysis, and secondary transport, in which H^+ gradient made by H^+ pumps is used as driving force to transport molecules. The former is managed by ATP-binding cassette transporter, whereas the latter is mediated by divergent membrane proteins of different types. For the latter, in particular, a remarkable progress has been made in MATE (multidrug and toxic compound extrusion)-family transporters, which seem to be deeply involved in accumulation and translocation of various natural products. In the congress, an overview of membrane transport mechanism for secondary metabolites as well as recent novel findings in this field are introduced, and the possibility to increase the productivity of valuable secondary metabolites by transport engineering in plants is discussed.

Challenges for the production of recombinant pharmaceuticals in plant expression systems

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Many of today's pharmaceuticals are derived from plants, but protein drugs are exceptional because they are predominantly of mammalian origin. They are usually produced in cultured human or rodent cells, which provide an acceptable biochemical environment. The biopharmaceutical industry has therefore evolved to view mammalian cells as the gold standard for production. The focus on mammalian cells has had a negative impact on the use of plants, despite their many potential advantages including the prospect of inexpensive, large-scale biopharmaceutical production without sacrificing product quality or safety. The first plant-derived pharmaceutical products have now been approved but these represent a tiny proportion of the products in development, products which could have a profound impact on the cost and availability of medicines to those most in need. The obstacles currently preventing the rapid commercialization of plant-based expression platforms are both technical and political. Overall, it is the technical hurdles which are falling the most quickly, since these revolve around two major issues – quality and quantity. Recombinant proteins produced in plant cells are identical to those produced in mammals, although there may be subtle differences in glycan structure. Such quality aspects are being addressed by protein targeting and host cell re-engineering to prevent the addition of plant glycans. The quantity of recombinant protein produced in plants is improving all the time, through the development of strategies to increase yields and protein stability. The major current challenge is to encourage the uptake of this technology, which will be more acceptable once a robust regulatory framework has been adopted, a goal being pursued by major academic and industrial partnerships in Europe and North America.

Production, recovery and bioequivalence of human insulin obtained from transgenic oilseeds

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The production of biopharmaceuticals in plants has been under investigation since 1985. Despite the wealth of technology available to exploit plants in this way, there is still no plant-made recombinant pharmaceutical protein that has been approved by a regulatory agency as a drug. Interestingly, this has not been because of excessive barriers imposed by regulatory authorities, but rather because no company or institution has yet pursued a molecule through all the necessary phases of clinical trials. In this presentation, I shall describe our progress in the manufacture of human insulin in Safflower seeds. This will include data on expression levels and cellular compartmentation, chemical and biochemical characterization of the plant-derived insulin product and the demonstration of its authenticity *in vitro* and *in vivo*. I shall further describe the regulatory path for this molecule, which in Europe is considered as a follow-on biologic, and the clinical work necessary to achieve approval of this plant-derived biopharmaceutical. The role of this effort in filling the gap between supply and demand for insulin worldwide will also be analyzed.

Preventing HIV with transgenic plants

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HIV continues to be a pandemic; nearly 50 million people are infected and more than 25 million people have already died from the disease. Efforts to curtail this disease are focused on the development of a vaccine as well as microbicides, which are products that can be applied to the vagina or rectum prior to intercourse to prevent the sexual transmission of HIV.

Plants offer significant advantages for the production of HIV vaccines and microbicides. Firstly, the scale of production required to make a global impact on HIV may only be practical by using transgenic plants as an expression system. For example, monoclonal antibodies for use as microbicides would be required at levels of 1000's kg per year. The growth of transgenic plants on an agricultural scale could provide virtually limitless opportunities for expansion.

Secondly, plant derived pharmaceuticals benefit from a low initial investment and current estimates demonstrate that plants offer significant economic savings compared to conventional fermentation techniques. This is of particular importance when addressing the HIV pandemic where developing countries bear the brunt of the disease but cannot afford the cost of many modern medicines.

Thirdly, the eukaryotic nature of plant cells allow the efficient assembly of complex molecules, this again has implications for HIV prevention strategies as a successful product is likely to depend on cocktail of antigens and peptides.

Here we describe progress on the production of HIV antigens and microbicides in transgenic tobacco plants, with an emphasis on monoclonal antibodies and cyanovirin-N as microbicide candidates. We demonstrate that such microbicide candidates can be efficiently produced in plants at levels approaching commercial feasibility.

Production of recombinant allergens in plants

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The routine diagnostic set-up for atopic allergies includes detailed documentation of the clinical history, provocation tests in skin or other target organs, and serology (laboratory tests for total and allergen-specific IgE antibodies). Since decades, skin tests and other provocation tests, as well as allergen immunotherapy are performed with extracts from natural sources. Presently, these extracts are standardized for their content of certain major allergens, a prerequisite for the production of consistent preparations. However, products prepared from natural sources are very heterogeneous and contain many allergenic and non-allergenic proteins, and other substances. Thus, the replacement of extracts by selected recombinant allergens is an emerging strategy for improving allergy diagnosis and immunotherapy. In this respect, recombinant production based on plant systems offers a number of advantages such as appropriate post-translational modifications and enhanced safety due to absence of animal or human pathogens. So far, several approaches to express allergens using plant systems have been published. Immunologically active Der p 2, a major house dust mite (HDM) allergen, has been expressed in BY-2 tobacco suspension cell cultures. Mal d 2, a thaumatin-like allergenic protein from apple, and Bet v 1, the major birch pollen allergen, both have been overexpressed in *Nicotiana benthamiana* using a tobacco mosaic virus (TMV) vector. The major mugwort pollen allergen Art v 1 has been expressed in both TMV and *Agrobacterium*-transformed tobacco plants.

Other approaches have explored the possibility of using seeds of cereal crops as vehicles for production of recombinant allergens and as direct delivery system without the need of allergen extraction and purification. Prototypes of such edible vaccines have been produced for a major HDM allergen Der f 1 in *Lotus japonicus*, and for T cell epitopes of Japanese cedar pollen allergens in rice seeds.

Optimizing glycosylation of recombinant proteins in plants

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Plants have the potential to become cost effective and safe factories for the production of recombinant therapeutic proteins, particularly when relatively large volumes are required. Yet, the range of applications is limited by the atypical *N*-glycan composition of plant-derived proteins due to differences in the biosynthesis of *N*-linked glycans between plants and mammals. In our lab we perform research with the aim to tailor *N*-linked glycosylation in plants towards specific glyco-products as well as to increase glycoform homogeneity.

The human *N*-acetylglucosaminyltransferase III (GnT-III) and ß1,4-galactosyltransferase 1 (GalT) genes were expressed in tobacco plants to study their effect on *N*-glycosylation of plant-made antibodies. The introduction of the GnT-III gene into tobacco caused highly efficient synthesis of bisected *N*-glycans on leaf glycoproteins as determined by MALDI- and LC-MS/MS analyses. Moreover, we have shown that the majority of *N*-glycans of a mAb produced in a plant expressing GnT-III was bisected, but still contained potentially immunogenic core-bound xylose (Xyl) or fucose (Fuc) residues.

In contrast, expression of a hybrid enzyme (xylGalT), consisting of the N-terminal domain of *Arabidopsis thaliana* xylosyltransferase (XylT) and the catalytic domain of GalT in tobacco produced a sharp reduction of *N*-glycans with core-bound xylose or fucose. A mAb purified from leaves of plants expressing xylGalT displayed an *N*-glycan profile featuring high levels of galactose, undetectable Xyl and a trace of Fuc. Hence, a transgenic plant expressing the hybrid GalT might yield more effective and safer monoclonals for therapeutic purposes than wild-type plants and even transgenic plants expressing the unchanged GalT.

Finally, strategies will be discussed that influence glycoform homogeneity. These strategies include culturing, choose of plant and plant tissue, as well as genetic approaches.

Second generation expression platforms for the high-yield production of proteins

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The potential of 'molecular farming', production of recombinant pharmaceutical proteins using plants or animals as bioreactors, is being illustrated by a number of recombinant protein products that currently undergo clinical trials. All these product candidates have been obtained using expression processes developed during late 1970ies – early 1980s, and all currently available 'first generation' expression methods suffer from various limitations, such as the long time frame necessary for stable transformation, the low yield obtained with stable or transient systems, biosafety concerns around open field cultivation of transgenic crops expressing therapeutic proteins, and the inability of transient systems to be scaled up. These available production platforms fall into several categories: nuclear transformation, plastid transformation, transient expression based on *Agrobacterium*-mediated delivery, and transient expression mediated by plant viral vectors.

The new generation process which we term 'magnifection', is a simple and indefinitely scalable protocol for heterologous protein expression in plants, which is devoid of stable genetic transformation of a plant, but instead relies on transient amplification of viral vectors delivered to multiple areas of a plant body (systemic delivery) by *Agrobacterium*. This eclectic technology combines advantages of three biological systems: vector efficiency and systemic delivery capabilities of an *Agrobacterium*, speed and expression level/yield of a virus, and posttranslational capabilities and low cost of a plant. The transgenic version of the technology relies on the integration of the proreplicon on a plant chromosome and its activation upon chemical induction. It is especially appropriate for the manufacturing of molecules where the cost of goods rather than the manufacturing speed is the limitation. Thus, the magnifection platform effectively addresses most of the major shortcomings of earlier plant-based technologies.

Molecular pharming in cereal crops and the political dimension of plant-made pharmaceuticals

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Plant-based systems are now practical alternatives for the production of recombinant proteins for medical or veterinary applications. Over the past two decades, a plethora of recombinant pharmaceutical proteins have been expressed in a range of plant species. These include some of the major crops such as corn, soybean and rice, and also tobacco and alfalfa. Impressive advances in terms of our understanding of how recombinant proteins accumulate in specialized tissues or organs, and molecular, biochemical and physiological factors influencing levels and stability of expression have all contributed to making plant-based production hosts competitive with fermenter-based systems and transgenic animals.

Despite such successes little has been achieved in translating fundamental science discoveries in a laboratory setting to commercial application. We will review the state of the art in molecular pharming, with emphasis on the production of medicinally important proteins in crop species, focusing on cereal crops. We will then review remaining constraints hindering the transition from the laboratory to the market and we will also discuss how political factors and an overburdening regulatory system have contributed towards a stifling environment for innovation and commercial success. We will contrast industrialized countries which may have alternative means to address medical problems, with developing countries which desperately need safe, effective and affordable medicines.

Plant cell cultures for the large-scale production of pharmaceuticals

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Plants have been part of the human pharmacopeia since the dawn of human civilization and over the period of evolution of the modern pharmaceutical industry have been an important source of drugs. Many live saving drugs highlighted by long standing applications in pain and oncology have been discovered from plants. Although plant based small molecule drug discovery was once the staple of big pharma and other research institutions, it has been replaced by leads generated from combinatorial chemistry or other natural product sources or through synthetic leads inspired by natural products. Problems typically associated with commercialization of complex molecules from plants such as; repeated access to biological material following discovery, overcoming natural variability and ability to efficiently and economically produce adequate drug supplies for clinical and commercial purposes, have been overcome through the application of plant cell culture technology. More recently, plants, by virtue of their being advanced eukaryotic hosts, have also become serious contenders for the expression of recombinant proteins for human pharmaceutical and other uses. Significant advances have been made in overcoming biosynthetic differences between plants and mammalian cells through pathway engineering and productivities of key therapeutic proteins are approaching commercially attractive levels. Plant cell cultures as liquid suspensions are an eminently scalable format for the large scale production of pharmaceuticals. The principles used for other cell based systems are applicable to plant cell culture as well. The economics and time scales of plant cell culture cultivation however demand judicious selection of targets and set the performance benchmarks for commercial production of valuable products. Time from discovery to manufacturing is also a key consideration. In this presentation, two case studies are discussed – one of large scale paclitaxel production and the other, the production of recombinant proteins, using plant cell cultures.

Design of bioreactors suitable for plant cell and tissue cultures

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Plant cell and tissue cultures are potential sources of secondary metabolites and recombinant proteins. In contrast to traditionally grown "whole wild plants" or "whole transgenic plants", their production in bioreactors guarantees defined controlled process conditions and therefore minimizes or even prevents variations in product yield and quality, which simplifies process validation and product registration. In order to achieve biomass productivities above 1 g dry weight L^{-1} d^{-1} coupled with moderate metabolite or protein levels, an optimized and well characterized bioreactor is required. Hence studies of the distinctive features of different bioreactor types (such as flow, shear pattern, mixing efficiency and oxygen transfer) are required if mass transfer and shear stress limitations, culminating in low cell growth and product yields, are to be reduced. Above all, the morphology, rheology, shear tolerance, growth and production behaviour of the culture should be taken into account when choosing the most suitable bioreactor type. Because these most relevant culture characteristics differ for mainly used plant cell suspension cultures and hairy roots, we distinguish between bioreactors for plant cell suspension cultures and bioreactors for hairy roots.

Stainless steel stirred bioreactors, bubble column reactors and airlift reactors directly derived from microbial bioreactors are commonly used with only minor modifications to grow plant cell suspension cultures up to 70 m^3 culture volume. Engineering analyses demonstrate that serious shear-related effects for plant suspension cells generally arise from the aeration and mixing system, the aeration rate and/or impeller tip speed used. Moreover, the superiority of low-cost and disposable bioreactors possessing a gas-permeable cultivation bag of plastic film was effectively proved in a number of plant cell suspension cultivations. Whereas Osmotek's Life-Reactor, Nestlé's Slug Bubble Bioreactor and Curtis' Plastic-lined Bioreactor represent pneumatically driven bubble columns, the BioWave was the first mechanically driven bag bioreactor. Today further examples of such bag bioreactors characterized by wave-induced motion are available, including, the Wave & Undertow Bioreactor, AppliFlex, Tsunami-Bioreactor, Optima and OrbiCell.

The physiology and morphology of hairy roots demand special consideration with regard to effective bioreactor design providing a low-shear environment for growing hairy roots and reducing mass transfer limitations in densely packed root beds. Immobilization of hairy roots by horizontal or vertical meshes as well as by cages or

polyurethane foam demonstrably promotes their growth in submerged stirred bioreactors, bubble columns, airlift reactors and drum reactors, where the roots are immersed in the culture medium. On the other hand, oxygen transfer limitation can be reduced or eliminated by growing hairy roots in emerged bioreactors, such as spray or droplet reactors and mist reactors, in which the roots are exposed to humidified air or a gas mixture and nutrients are delivered as droplets by spray nozzles or ultrasonic transducers. In addition, hybrid bioreactors were developed to guarantee simple and safe hairy root inoculation. Encouraging results, which were achieved in Life-Reactor, Plastic-lined Bioreactor and BioWave allow the conclusion that low-cost disposable reactors may be also interesting alternatives for growing hairy roots up to large-scale.

Large-scale production of plant cells for cosmetic applications

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The cosmetic environment is the third area next to food and pharmacy where plants and plant derived products play a very important role. Most cosmetic products and their application are defined by active ingredients. Those active ingredients may derive from either synthetic sources or from plant sources. No other origin like human or animal is accepted or allowed in cosmetics nor are genetically modified plant sources. The whole cosmetic research and development society is desperately seeking new innovative plant ingredients for cosmetic application. At the beginning native traditional medicinal plants were used in the cosmetic field then the exotic plants from Amazonas region or African sites were harvested equally the traditional Chinese medicinal plants or Ayurvedic plants came into cosmetics. Then the cosmetic developers expanded their field of research from earth to sea and used marine derived plants. What comes next? Where to find other plants with cosmetic activity? Several plants of cosmetic interest are not to be used due to following facts: the plants contain toxic metabolites, the plants grow too slowly and a seasonal harvesting is not possible, the concentration of plant constituents differ from harvest to harvest or the plant is endangered and not allowed to harvest. With the plant cell culture technology we bring complete new aspects in development of novel cosmetic plants derived actives.

Biotechnology as well as plant cell culture technology is not yet common in cosmetic field. But the plant cell culture technology may help to overcome essential problems in manufacturing of cosmetic products: Plant raw material as a biological product for metabolites is not always reproducible in quality and quantity. With cell cultures the dependence of seasonal harvesting has disappeared. There is no concern necessary about endangered plant species. The batch to batch differences in plant components are eliminated. The other thing is that plant cells in culture are able to produce higher concentrations of specific metabolites (antioxidants or proteins) through eliciting factors like UV radiation, jasmonic acid or toxic substances. This enables to reach a higher product yield in manufacturing active ingredients. Due to all these findings, we decided to risk the step in plant cell culture derived cosmetic active ingredient production. We started the project in collaboration with the University of Wädenswil and expanded the project from laboratory scale to production scale. To prevent the risk of contamination and to reduce cleaning costs, we decided to use disposable reactor systems to cultivate the plant cells. To obtain a suitable cosmetic product we used the high pressure homogenization technique to decompose the plant cells and release all the beneficial constituents while we encapsulate these components at the same time in liquid Nanoparticles.

Bioreactors for Plant Science: Production of ingredients and/or somatic embryos of improved species?

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Abstract not available

Where do we go from here: The next decade of plant biotechnology

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Forecasting is always difficult, especially for the future. For a longer run, technology of the future will feel like magic to us. We can ask, what does today's technology feel like, if we look at it with eyes a decade old. *Arabidopsis* was not sequenced, but we knew it was coming. Microarrays had just emerged, but we did not know how they would explode the data flow concerning plant gene expression analysis. Sequencing had gone robotic, but we did not see massive parallel sequencing. If not quite magic, at least dreams come true.

Plant biotechnology follows the path of plant molecular biology. Genomics and systems biology will show the light in the near future. For example, we have already seen how integrated metabolic engineering is built. But surprisingly, if we look at agricultural biotechnology – the transgenic crop plants that grow in the fields – nothing has happened in ten years. Our herbicide resistant and Bt crops of today are products of biotechnology that is two decades old. Could we foresee the power of public opinion?

In the next ten years, plant biotechnology and plant breeding will approach each other. Transgenic plants expressing exotic foreign genes will stay as special solutions, but the main impact will be done using non-exotic transgenes – essentially plant's own genetic material – or methods that mimic natural mutations. Will this change the public opinion and finally open possibilities in using knowledge-based methods in developing new cultivars? There are some signs that it will not. We will need something else in the post-genome era. Plants for human health may be the key.

SHORT LECTURES
S1–S4

Sesquiterpene synthases: Key enzymes in the biosynthesis of medicinal sesquiterpenes

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Sesquiterpenoids are a structurally diverse class of isoprenoids found in plants, some fungi, and bacteria. The structural diversity and stereochemical complexity of sesquiterpenoids are remarkable. More than 300 types of cyclic sesquiterpenes have been characterized to date and all are derived from a common acyclic precursor, farnesyl diphosphate (FPP), in a reaction catalyzed by a sesquiterpene synthase. Sesquiterpene synthases perform critical biosynthetic tasks in metabolic pathways. A number of sesquiterpenes are used (*e.g*. artemisinin from *Artemisia annua*) or have potentials as pharmaceuticals. We are involved in studies on the biosynthesis of such compounds. We have cloned a number of sesquiterpene synthase from different plants. The recombinant proteins have been produced in *E. coli*, purified and characterized. A (general) method to clone sesquiterpene synthases from plants by using 3'-RACE and 5'-RACE systems was developed. We will describe the cloning, recombinant expression and characterization of a number of sesquiterpene synthase from different plants.

When expressing in a bacterial system, the various sesquiterpene synthases behave very different. The tendency to form inclusion bodies varies. A relatively simple method to optimize the expression of soluble sesquiterpene synthases in the bacterial host was developed. Deuterium-labeled FPPs to elucidate the mechanism by which amorpha-4,11-diene synthase and germacrene D synthase carry out the cyclization reaction was used. The effects of fusion of two enzymes on the metabolic flow have been studied. FPP synthase, producing the substrate for sesquiterpene synthases, was fused to *epi*-aristolochene synthase, a sesquiterpene synthase from tobacco. The recombinant bifunctional enzyme was studied. Finally, amorpha-4,11-diene synthase has been transformed into yeast as a first step to develop a biotechnological process for the production of artemisinic acid, a precursor of artemisinin, which can chemically be converted to artemisinin.

Molecular approach to the regulation of the production of taxol and other taxanes in cell cultures of *Taxus baccata*

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Plant cell and organ cultures constitute a promising future for the production of numerous valuable secondary compounds, although efforts in this field have so far had limited commercial success. Empirical approaches have long been employed for the development and optimization of plant cell-based bioprocesses, focusing on input (cell line, medium, culture parameters, bioreactors, process operations, etc.) and output factors (cell growth, nutrient uptake, productivity, yield, etc.). In this context, we have developed hairy root cultures of *Panax ginseng*, engineered root cultures of *Duboisia*, *Datura metel* and *Hyoscyamus niger*, and plant cell cultures of *Centella asiatica, Ruscus aculeatus* and *Taxus* spp.

Paclitaxel is a compound with antitumoral activity that is extracted from *Taxus* (yew) species. The difficulty in obtaining this compound from yew trees has limited its clinical use. In order to develop a superior biotechnological system for high paclitaxel production we have optimized *in vitro* culture conditions by assaying several basic media, plant growth regulators, sugar supplements etc. As secondary metabolite production in plant cell cultures usually does not depend on growth *per se*, a two-stage culture system has been established. Plant cells are first cultured in a medium optimised for growth, which is subsequently replaced by a production medium that stimulates secondary metabolism. This system has an added advantage of permitting the addition of biosynthetic precursors and elicitors when secondary metabolite production reaches highest levels, i.e., during the culture's second stage. Studies have shown that paclitaxel production in *Taxus* cell cultures is clearly further stimulated by the addition of methyl jasmonate (MeJA) to the production medium.

In a rational approach towards the elucidation of paclitaxel production, we are studying its metabolism, and considering how different culture factors affect metabolic and gene expression profiles in *Taxus baccata* cell cultures. Thus far, we have studied transcription profiles in *Taxus* cell cultures, elicited or not with MeJA, by genome-wide cDNA-AFLP analysis and we have identified a number of genes of which the expression correlates with enhanced production. Among them, there are genes that code for enzymes directly related with paclitaxel biosynthesis and others that code for transcription factors. Currently, we are further functionally analysing these genes, in order to assess their precise role in the regulation of paclitaxel biosynthesis, by means of various approaches, such as transient and stable overexpression analysis and *in situ* hybridisation.

From planta to pharma with glycosylation in the toolbox

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The plant-specific glycosylation was long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy. Our goal here is to emphasise all the progress recently made towards humanization of *N*-glycosylation in plants, and to illustrate that plant typical *N*- and *O*-glycosylation progressively emerge as additional advantages for using this promising expression system. We have recently cloned and characterized several glycosidases and glycosyltransferases responsible for plant *N*-glycan maturations. Our studies on signals and mechanisms responsible for the distribution of this machinery along the plant secretory pathway have shown that glycosylation enzymes are organized according to an assembly line distributed in four domains, the ER, the ER / early Golgi compartment, the medial and late Golgi apparatus[1].

Some glycosylation enzymes such as mannosidase I are located exclusively based on the length of their transmembrane domains, some others such as glucosidase I contain at least three different signals located in the cytosolic tail and stem of this type II ER membrane protein, each signal being independently sufficient for ER retention of a reporter protein. Plants have the capacity to synthesize highly complex heterologous proteins and are able to perform most post-translational maturations, including *N*-glycosylation, required for a plant-made human protein to be biologically active[2]. However, structural characterization of several plant-made pharmaceuticals (PMPs) has shown that like any other heterologous expression system, plants are unable to reproduce a human type *N*-glycosylation[3]. We are currently developing knock out and knock in strategies to humanize *N*-glycosylation in plants, including reconstruction of the human *N*-glycan sialylation pathway *in planta*[4] and the use of targeting signals identified in plant glycosylation enzymes for a targeted expression of human glycosyltransferases in the plant secretory pathway.

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Transgenic plants for animal health: Edible vaccine against piglet ETEC diarrhea

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F4 fimbriae are the major colonization factors associated with porcine neonatal and postweaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC). Via the chaperone/usher pathway, the F4 are assembled as long polymers of the major subunit FaeG, which also possesses the adhesive properties of the fimbriae. Being highly stable and mucosally immunogenic F4/FaeG offers a unique model system to study oral vaccination against ETEC-induced postweaning diarrhea (PWD). PWD is a major problem in piggeries worldwide and results in significant economic losses. No vaccine is currently available to protect piglets against PWD.

Transgenic plants provide an economically feasible platform for large-scale production of vaccine antigens for animal health. Here, the capacity of transgenic plants to produce FaeG was evaluated. Using the model plant tobacco, FaeG was directed to different subcellular compartments by specific targeting signals. Targeting of FaeG into chloroplasts offered a superior accumulation level of 1% of total soluble proteins over the endoplasmic reticulum and the apoplast. Intrinsically, the incomplete fold of fimbrial subunits renders them unstable and susceptible to aggregation and/or proteolytical degradation in the absence of a specific periplasmic chaperone. The chloroplast-targeted FaeG was purified from tobacco and crystallized. The crystal structure shows that chloroplasts circumvent the absence of the fimbrial assembly machinery by assembling FaeG into strand-swapped dimers. Moreover, the FaeG-dimers retained the key properties of an oral vaccine, i.e. stability in gastrointestinal conditions, binding to porcine intestinal F4 receptors, and inhibition of the $F4^+$ ETEC attachment to F4R.

To investigate the oral immunogenicity, the FaeG protein was expressed in the crop plants alfalfa and barley. Desiccated alfalfa plants and barley grains stored FaeG in a stable form for years. When the transgenic alfalfa plants and cholera toxin were orally

co-administered to weaned piglets, F4-specific systemic and mucosal immune responses were induced and the duration and number of $F4^+$ *E. coli* excretion following $F4^+$ ETEC challenge were reduced. In conclusion, these results suggest that transgenic plants producing the FaeG subunit protein could be used for production and delivery of oral vaccines against porcine PWD.

ABSTRACTS OF POSTERS

EMERGING TECHNIQUES
A1–A8

Analysis of human gut model metabolites by GCxGC-TOF

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Dietary phenolic compounds are plant derived secondary metabolites, which can be divided into flavonoids, phenolic acids, stilbenes, tannins and lignans. Catechins can be found as monomers e.g. in tea or as condensed forms, proanthocyanidins (PA), in bilberries, apples, grapes and beverages derived from those fruits. PAs are formed from (+)-catechin and (-)-epicatechin units. Phenolic compounds are ubiquitous metabolites that are metabolised extensively during uptake. It is possible that a major proportion of the metabolites are formed in the colon by microbiota. For example lignans are converted to enterodiol and enterolactone, flavonols are converted to phenylacetic acids and flavanols and flavanones to phenylpropionic acid derivatives. These metabolites, predominantly small phenolic acids, have been identified in human plasma and urine. The residence time of the colonic metabolites is longer than the original structures from plant foods. Epidemiological studies have shown that high concentration of enterolactone is associated to lowered risk of chronic diseases like cancer and cardiovascular disease. Similar evidence for flavanoids and other phenolic compounds is lacking. Identification of diverse flavonoid metabolites is needed.

This work aims to compare the *in vitro* microbial metabolism of (+)-catechin and (-)-epicatechin units using human faecal microbiota as an inoculum to identify their main degradation products. Pure (+)-catechin and (-)-epicatechin were fermented with pooled human faecal microbiota in strictly anaerobic conditions. The sample preparation steps include extraction with ethyl acetate and silylation with MSTFA. GCxGC-TOF analytical method was developed for the analysis of the microbial metabolites. We will describe the method including data processing steps and the preceding sample preparation steps for the analysis of microbial metabolites of (+)-catechin and (-)-epicatechin. The compounds include e.g. hydroxyphenyl propionic and acetic acid derivatives.

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Secondary metabolism in strawberry receptacle and achenes during development revealed by metabolic profiling using UPLC-qTOF-MS

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Metabolomics plays an essential role in the survey of natural product resources of plants. GC-MS and LC-MS are among the key technologies applied for metabolites detection and identification. The performance and applicability of MS techniques have advanced rapidly, and extensive datasets of high mass-accuracy information of phytochemicals can be generated. In this study we used a high resolution qTOF-MS system to evaluate the metabolite composition of strawberry fruit, a rich source of secondary (or specialized) metabolites. Non-targeted metabolic profiling was conducted in two strawberry fruit tissues (achenes and receptacle) in six different stages of fruit development. Methanol extracts were analyzed by UPLC-qTOF-MS in both the positive and negative ionization modes and the raw data was first processed for peak picking across samples by the MarkerLynx program (Waters Inc.). The data was subsequently analysed to identify the different metabolites produced during development of these two tissues. One major issue in non-targeted LC-MS metabolite analysis is that a number of metabolites together with their fragments may elute at the same retention time. To distinguish between different metabolites in a certain retention time window we developed a computer programme that could aid in grouping mass signals belonging to a single metabolite. The markers were grouped based on the correlation of the abundance profiles of each mass signal in the different sample types and developmental stages. After grouping the markers across the entire chromatographic run, the data was further processed to cluster metabolites according to their expression profile and identify the metabolites using accurate mass information. Clustered metabolites belonging to compound classes including flavonols, phenolic acids, proanthocyanidins and ellagitannins were identified in both receptacle and achene tissues and their accumulation during development was determined.

NMR metabolomics of jasmonic acid and pectin treated *Cannabis sativa* L. cell suspension cultures

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Cell cultures of medicinal plants are an alternative and promising source of bioactive compounds for medicinal use and they are also a good model system to investigate metabolic pathways. *Cannabis sativa* L. is an annual dioucius plant from the family Cannabinaceae, with interesting pharmacological effects like antiemetic, antiglaucoma and analgesic. Metabolomics and other -omics methods can help to better understand regulation of metabolic pathways in order to modify or improve the production of specific compounds.

NMR metabolomics was used to investigate crude extracts of two suspension cultures of *C. sativa* "Fourway" initiated from leaves. High resolution NMR was used to identify and quantify the production of metabolites during a time course of the suspension cultures, elicited with jasmonic acid or pectin. Statistical evaluation using Principal Component Analysis (PCA) was used for the mixture analysis of ^{1}H NMR spectra from methanol-water and chloroform fractions. Media of the cultures were also analyzed. For the structure elucidation we used 2D NMR (J-resolved, COSY, HSQC and HMBC).

These studies were focused on the analysis of primary metabolites and secondary metabolites like phenylpropanoids, cannabinoids and terpenoids. In the polar phase mainly primary metabolites were identified such as the amino acids asparagine, threonine, tryptophan, and sugars (e.g. glucose and sucrose). Moreover it was observed that tyrosine, phenylalanine and a 1, 4 disubstituted benzene analogue make the separation by PCA from elicitation with jasmonic acid. The non polar fraction revealed the presence of fatty acids, lipids and terpenoids. Jasmonic acid showed greater effect on the cell metabolism than pectin.

NMR-non-targeted metabolite profiling to reveal the function of a transcription factor involved in defence response of tobacco

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Recent discoveries have shown that the expression of secondary metabolites is under transcriptional control. So far, studies on transcription factors using transgenic approaches may unravel control mechanisms of secondary metabolism operative in the defence response of plants. The ultimate goal of -omics sciences is to acquire an integrated understanding of biology. The metabolomic-based approach, to define the chemical phenotype of an organism, is regarded as a direct way to depict the picture of the whole of biochemical processes underlying the *status* of a certain system. Metabolomics can thus reveal the function of genes involved in metabolic pathways. As analytical tool to describe the entire metabolome of an organism, physical measurements, like spectrometric techniques, represent unique, ever-lasting and reproducible methods. We chose an NMR-metabolomics based approach for the analysis of transgenic *Nicotiana tabacum* plants with perturbed defence metabolism. A specific subset of the tobacco pathogenesis-related proteins (PR proteins), the PR-1, is triggered by exogenous application of the plant hormone salicylic acid and is now generally regarded as marker for systemic acquired resistance (SAR). SAR is a broad spectrum resistance to viral, bacterial and fungal pathogens in distant, uninfected plant parts. During previous experiments in our lab, a cDNA clone was isolated encoding a peptide that specifically binds to the transcription start site of the *PR-1a* gene (data not published). The corresponding protein (NtPBP1) appeared to be a member of the well conserved set of plant DNA-binding proteins known as WRKY proteins. Experiments have demonstrated that NtPBP1 functions as a transcription factor. In order to elucidate whether the protein is indeed involved in inducible expression of the *PR-1a* gene, and to determine the possible consequences of its manipulation, experiments have been conducted to overexpress NtPBP1 in tobacco plants. The resulting transgenic plants have been analyzed by means of NMR, and the data processed by multivariate data analysis (MVA) in order to elucidate the pathways affected and to link the expression patterns of genes connected with the systemic acquired resistance.

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Metabolomic characterization of *Brassica rapa* leaves by NMR spectroscopy

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The *Brassica* genus has been extensively studied due to the nutritional properties and beneficial effects on health. However, the amount of species, varieties, and cultivars included in this genus and the resulting large metabolic variation has been an obstacle for the systematic research of plants of this genus. In order to overcome the problems posed by the biological variation, the metabolomic analysis of various cultivars of *Brassica rapa* was performed by NMR spectroscopy combined with multivariate data analysis. Discriminating metabolites in different cultivars and between different stages of development were elucidated by diverse two-dimensional NMR methods after sorting out the ^{1}H-NMR signals that differentiate the samples by principal component analysis. Among the elucidated metabolites, several organic and amino acids, carbohydrates, adenine, indole acetic acid (IAA), phenylpropanoids, flavonoids and glucosinolates were found to be the metabolites contributing to the differentiation between cultivars and age of *Brassica rapa* plants.

Metabolomic approach to identifying bioactive compounds in berries: Advances toward fruit nutritional enhancement

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Plant polyphenolics continue to be the focus of attention with regard to their putative impact on human health. An increasing and ageing human population means that the focus on nutrition and nutritional enhancement or optimization of our foodstuffs is paramount. Using raspberry as a model we have shown how modern metabolic profiling approaches can be used to identify the changes in the level of beneficial polyphenolics in fruit breeding segregating populations and how the level of these components are determined by genetic and/or environmental control. Interestingly the Vitamin C content appeared to be significantly influenced by environment (growth conditions) whilst the content of the polyphenols such as cyanidin, pelargonidin and quercetin glycosides appeared to much more tightly regulated suggesting a rigorous genetic control. Preliminary metabolic profiling showed that the fruit polyphenolic profiles divided into two gross groups segregating on the basis of relative levels of cyaniding-3-sophoroside and cyaniding-3-rutinoside. These compounds are implicated as conferring human health benefits.

Mechanisms related to plant metal homeostasis and accumulation

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Some heavy metals like Cd, Pb, Hg are very toxic for living organisms even in very small quantities, whereas many transition metals (e.g. Cu, Zn, Fe) are indispensable micronutrients. There is a lot of local variation in the metal concentrations and both deficiency and toxic levels exist. High concentrations in soils pose a significant risk due to uptake by plants and intake by humans and animals. On the other hand, low Zn is a limiting factor for the production and quality of cereals and also one of the most serious micronutrient deficiencies in humans.

Plants have evolved a range of mechanisms that control the uptake and accumulation of both essential and nonessential metals. Despite the recent advances in metal homeostasis research, the molecular mechanisms related to metal homeostasis network in plants are practically unidentified. If those mechanisms would be better known, they might offer possibility to affect the bioavailability of metals and their concentration in plants for production of healthier nutrition.

One of the particularly interesting model plants to study the metal-associated characteristics is the metal hyperaccumulator *Thlaspi caerulescens*, a close relative to *Arabidopsis*. *Thlaspi* can collect exceptionally high concentrations of Zn and Cd in its shoots. It also has several accessions with a wide variety of metal uptake and translocation characteristics, offering thus a good model for the metal homeostasis research.

In our studies we have compared proteomes of metal-exposed *Thlaspi* accessions using two-dimensional electrophoresis (2-DE). Differences in the protein patterns were mainly seen between the accessions, whereas metal exposures played a minor role. A number of proteins possibly related to metal accumulation were identified with the help of sequence data obtained from mass-spectrometric analysis and homology searches of databases. Contribution of the observed differences to the metal accumulation is being investigated further by comparing *Thlaspi* crosses. Also *Arabidopsis* mutants for the selected genes are being characterized and their role in metal homeostasis is being studied.

SoluCel's integrated approach for elucidation and engineering of secondary metabolite pathways

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Because of their excellent biological and chemical diversity, plants are an important source of high-value compounds. Plant secondary metabolites have applications in *e.g.* the pharmaceutical, agrochemical, fine chemical, personal care, cosmetic, flavor & fragrance and nutraceutical industries. The market of plant-derived pharmaceuticals alone already represents a value of >US$ 40 billion with important drugs as morphine, atropine, paclitaxel and vinblastine. It is estimated that ±25% of all pharmaceuticals are plant compounds or chemical derivatives thereof.

The full potential of secondary metabolites has not been realized as many potential products have not been developed due to high lead finding cost and unfavorable economics. Many secondary metabolites are economically not viable because of the high production costs caused by supply and quality problems from the natural plant source and the difficulty of cultivating the often rare species in which these molecules are found. Chemical synthesis is impossible or very costly due to the complex structures of secondary metabolites and plant cell cultures yield only low levels. Genetic engineering was seen as a solution to increase the levels of secondary metabolites, but this has proven to be more difficult than anticipated because the synthetic pathways are largely unknown and highly complex. In other words, the information is lacking for rational engineering of the biosynthetic pathways to increase metabolite levels.

SoluCel, a joint venture of VIB (Belgium) and VTT (Finland), is a biotechnology company that discovers, develops and produces plant-derived pharmaceuticals. In addition research services are offered. Both activities are based on SoluCel's unique technology platform that unlocks the vast economic potential of plant secondary metabolites by an integrated approach for elucidation and engineering of their pathways.

Applications of SoluCel's technology platform include cost-efficient production of secondary metabolites, development of metabolite and genetic markers for productivity or quality traits, biotransformation of for example unnatural substrates, knock-out of *e.g.* toxic or anti-nutritional compounds and generation of novel and/or improved secondary metabolites. The power of the technology platform was demonstrated by the elucidation of the terpenoid indole alkaloid pathway in Catharanthus roseus at the metabolite and transcript level. Almost all of the known, but more importantly many unknown, metabolites and genes could be identified in a single experiment. SoluCel possesses one of the world's largest collections of novel and known gene sequences of medicinal plants.

BIOACTIVITY
B1–B15

Evaluation of the anxiolytic like effect of the *Tilia americana* var. *mexicana* methanol extract in mice

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Tilia species have been used for their properties in traditional medicine around the world. In Mexico, the infusion of flowers is used to treat enterocolitis, gastroenteritis, migraine, some types of spasms, liver and gall bladder disorders, but mainly for nervous tension or insomnia. In this study, the anxiolytic and sedative effects of the methanol crude extract of *Tilia americana* var. *mexicana* were analyzed in two different experimental models (elevated plus-maze and hole board tests) in mice. Characterization and identification of the constituents in the methanol extract was made by HPLC analysis.

T. americana var. *mexicana* inflorescences methanol extract produced a significant and dose-dependent lengthening of the hypnosis time induced by sodium-pentobarbital in mice. This extract (10–300 $mg.kg^{-1}$, i.p. demonstrated a significant and dose-dependent attenuation in the anxiety-response in the plus-maze test at a 10 and 100 mg/kg dosage, and a diminution in the ambulatory activity and in the head-dipping behavior at higher doses (300 mg/kg) demonstrating a sedative effect. HPLC analysis of the methanol extract of *T. americana* var. *mexicana*, revealed that tiliroside, quercetin-*O*-pentosylhexoside, kaempferol-*O*-pentosylhexoside and quercitrin were the main flavonoid components as possible responsible of the activity in this species. Results of the present study show that *Tilia americana* var. *mexicana* possesses depressant activity on the central nervous system similar to the better-studied species of European *Tilia* and reinforces its use as anxiolytic and sedative in traditional medicine.

Functional oat fractions for healthy food products

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Oat is a superior source of soluble fibre plus phytochemicals, unsaturated fatty acids and high-quality protein. The consumers are aware of the health-promoting properties of oat, and the physiological effects of oat β-glucans are well-documented. Pre-treatments such as germination can be used to further increase the amount of some specific bioactive compounds. Different types of commercial β-glucan fractions are available on the global market, but the use of other potential oat fractions like oat protein, starch and oil is very limited at the moment. One reason for this is that the available fractionation processes of oat are not always technologically feasible and only some of the fractions meet the needed quality. A novel fractionation process has been developed for oat in order to improve the technological and functional properties of oat fractions including different types of high fibre and protein fractions, starch, oil and polar lipid fractions and a phenolic fraction. The benefits of these fractions and their applicability in health-promoting food products will be discussed.

In vitro and *in vivo* tests for to evaluate efficacy and toxicity of betulinic acid standalone or mixed with PVP

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Betulinic acid is a pentacyclic triterpene intensively analysed for its anti cancer selective effects on some tumour cells (eg. melanoma cells). Its activity has been determined analysis on a series of tumour cells like A 2058 (metastatic melanoma). The toxic activity was approved on human mesenchymal stem cells. *In vivo* tests consist in the analysis on embrionated egg model for to correlate the data. Materials and methods were specific for the *in vitro* and *in vivo* evaluations: microscopic and MTT tests plus spectroscopy analysis. The dissolution of the compounds in an aqueous environment (serum) was achieved by adding of PVP at 1:4 ratio as was indicated in literature data. DMSO at low concentrations was used for dissolution *in vitro* tests. The conclusions are that betulinic acid is active in low doses starting at 1 mg/ml on the tested tumor cells and has reduced toxicity (max. 40%). It determines an antiangiogenic effect on the embryonated eggs.

Technologies to increase the content of secondary phenolic metabolites in *Aloe* species

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During the 20 years more than 100 Aloe species originating from the desert of South Africa we have introduced to the Botanical Garden of the Jacob Blaustein Institute for Desert Reseach at Sade Boker in the Negev Desert of Israel. The *Aloe* plants have flourished in the desert conditions and are planted in loose soil. In addition to the average annual 100 mm of rain in this area in winter, they are irrigated with about 300–400 mm water.

Leaf exudates from *Aloe* species are used to great extent in traditional medicines. The succulent leaves of *Aloe* species have been used for medicinal purposes, cosmetic and food supplement, and also in gardening to save water as ornamental plants. Among the succulent leaves of *Aloe* plant species the secondary phenolic metabolites (SPhMs) were analyzed, including: barbaloin, homonataloin, nataloin, aloeresins and aloenins. SPhMs have been found to have strong inhibitory effect, antiinflamatory and catharactic effect *in vivo*. SPhMs are very important defending compounds against leaf eater in order to keep the natural balance in the green world. The SPhMs of *Aloe* plant are also involved in the protection of the plants from UV irradiation damage. The aim of this study is to present different methods, which were used to increase content of SPhMs in some *Aloe* species.

Tocopherol profile during drupe development in Italian olive cultivars

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The fruits of olive trees (*Olea europaea* L.) can be either processed as table olives or milled to produce olive oil. According to varieties, some of them are cultivated specifically for table consumption while the majority is used for oil extraction. Traditionally, the olive tree is grown mainly in the Mediterranean area, but the benefits of olive products on human health have been widely recognized and spread throughout the world.

Tocopherols are antioxidant compounds that play a key role in conferring nutritional value to olive fruits (drupes). Tocopherol biosynthesis takes place on the inner membrane of chloroplasts and chromoplasts. The four tocopherols, α, β, γ and δ-tocopherols, differ from one another by number and position of methyl groups in the phenolic part of the chromane ring. The multifunctional roles of tocopherols are related to their preventive action against reactive oxygen species in biological systems. In the past, α-tocopherol was considered as the isomer exhibiting the highest biological activity. Nevertheless, recent studies suggest that the other vitamin E isoforms have also important roles in the human organism. For example, γ-tocopherol has considered as a cancer chemopreventive agent and as a potent and effective agent in the prevention of cerebral infarction induced by middle cerebral artery occlusion. Thus, the knowledge of tocopherol profile is essential for estimating the potential antioxidant and biological activities of a specific food.

In this context, the aim of present study was to estimate the tocopherols profile in the drupes of nine Italian olive cultivars. During drupe development, several structural changes and chemical transformations occur that affect the different components of fruits. On this basis, tocopherol profile was monitored at different stages of fruit development. We demonstrated that tocopherol profile is changing according to cultivar and fruit ripening stage.

This research was supported by MIPAAF under RIOM-Project and OLIBIO Project.

Adopting biotechnological strategies – plants from South Africa as targets

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Healing through use of plants remains an inherent part of African communities but application of biotechnology to medicinal flora is still in its infancy. Although biotechnology by many is viewed as a 'high-tech' science with little relevance in the traditional medicines sector, South African researchers have awakened to the benefits of applying both micropropagation and transgenic technologies in order to add value to African medicinal plants. Aromatic medicinal plants, *Salvia africana-lutea* and *Pelargonium sidoides*, have been utilized as targets in our laboratories and will serve to highlight the benefits of a biotechnological approach to indigenous South African medicinal flora. Using clonal propagation, transgenic technologies and subsequent metabolite profiling through GCMS, LCMS and NMR, interesting changes in the chemical footprint of these plants have been elucidated. Extracts from transgenic cultures exhibit different chemical profiles compared to untransformed root cultures. Even so, their biological activity in pharmacological bioassays is surpassed by extracts from *in vitro* propagules of *Salvia* that are not transgenic. In this case, the tissue culture system may not only serve/facilitate conservation of these plants as they are popular as herbal products in the traditional medicines sector but the culture microenvironment also appears to induce *de novo* biosynthesis of a new subset of biologically active terpenoid compounds. Interesting pharmacological actions apparent in tissue culture-derived extracts and 'hairy root' cultures that are not indicated in the natural plant, are viewed as not only being important for the commercial sector but are proving to be a useful tool to gain insights to the control of secondary metabolism.

Development of a RP-HPLC method for chemical characterisation of *Coreopsis tinctoria* aqueous extract and screening for antidiabetic activity

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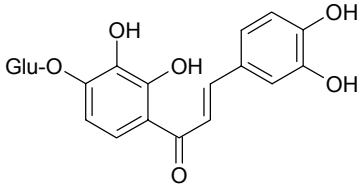
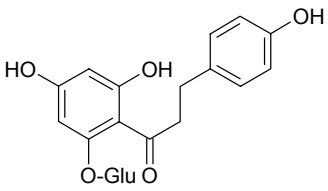
Coreopsis tinctoria Nutt. has been traditionally used in Portugal as an herbal drug for type 2 diabetes treatment.[1] The presence of flavonoids such as marein and coreopsin has been described for this species[2] and flavonoids are known to have antioxidant activity which can be important in the prevention of diabetic complications. Additionally, these two compounds are structurally similar to phloridzin, a classic SGLT1 inhibitor.[3] In order to characterize the aqueous extract, a phytochemical study was performed and marein was identified as the major flavonoid in the extract. In view of this, a simple and reproducible method for quantification of marein by RP-HPLC was developed and validated. The method showed to be linear (r^2> 0.99), precise (RSD< 4%), accurate (approximate recovery of 102%) and with appropriate limits of detection (LOD = 3.2 μg/ml) and quantification (LOQ = 9.8 μg/ml).

For the biological assays an infusion of *C. tinctoria* was prepared as recommended in commercial bags and this extract contained 4.6% (w/w) marein. The extract (25–300 mg/kg) showed no acute antihyperglycaemic activity using the Oral Glucose Tolerance Test, in rats, but showed antioxidant activity (EC_{50}= 21 μg/ml, DPPH method). The results of this study show that the quantification method developed is suitable for the chemical quantification of *Coreopsis tinctoria* aqueous extract. Concerning the antioxidant activity the results are in accordance with the expected for this type of compounds. Finally, the results of the acute antihyperglycaemic assay do not support the traditional use of this herb. We believe that there must be other mechanism than SGLT1 inhibition involved in the antidiabetic potential of this extract, namely through the antioxidant activity, and so other assays are in progress.



marein



phloridzin

The authors wish to thank the FCT-Portugal for the financial support.

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Argentina plants as potential sources of antitumoral compounds

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Human mammary adenocarcinoma is one of the most common malignant tumors worldwide. Surgical resection and chemotherapy are the most commonly used techniques for treatment. The development of new agents for breast cancer is important in order to reduce the mortality caused by this disease. Drug product discovery programs involves a number of different phases, such as selection and collection, extraction and biological evaluation, isolation and elucidation. The search for natural compounds with antineoplastic activity is one of the current priorities in the fight against cancer throughout the world. However, there is practically no information about species which have been used in the treatment of cancer. This is due to cancer being a complicated and heterogeneous illness, thus making evaluation of treatments difficult. Screening was carried out with organic solvents (petrol ether, dichloromethane, methanol) and aquoues extracts of: *Mandevilla laxa*, *Mandevilla pentlandiana*, *Aristolochia stuckertii, Gaillardia megapotamica*, *Heterothalamus alienus*, *Acalypha communis*, *Sebastiania commersoniana*, *Pteromonnina dictyocarpa, Urbania pappigera, Lantana grisebachii*, *Zexmenia bupthalmiflora* and *Baccharis sessiliflora* on MCF 7 (human mammary cancer) cell line.

The results showed that 10 of the 15 native plants used in this study presented cytotoxic activity (19 of the different extracts). The *T. megapotamicum* (Ether, MeOH and CI_2CH_2) extracts exhibited a pronounced cytotoxic effect (<25% of cell viability). A similar inhibitory effect was found with the MeOH of *L. nitida* and with the aqueous extract of *A. stuckertii*. The effects of the plant extracts studied in MCF-7 with paclitaxel at 0.1 μg/ml, it can be observed that this powerful antitumoral agent was transforming its morpological characteristic form during the time of incubation. At 3 hours, it displayed a fusiform structure, only forming an apoptotic morphologic cell at 20 hours. Meanwhile, after 3 hours of incubation with different extracts, the MCF-7 cells fell away from the walls of the well tissue culture test plates, indicating cell death.

Antileishmanial, antimalarial and cytotoxic activities of 12,1 dideoxy aegyptinone B from *Zhumeria majdae* Rech.f. & Wendelbo

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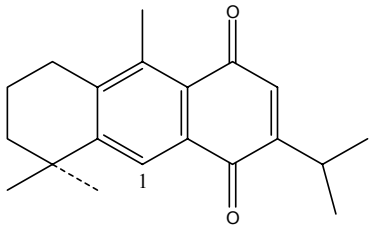
Zhumeria majdae (Labiatae) is an aromatic perennial shrub with beautiful flowers. It posses one species which is distributed in the northern heights of Bandar-Abbas, Hormozgan Province, Iran. For a long time, leaves of this plant have been used for stomachache and dysmenorrhea by the people of Hormozgan Province. During biological activities screening of some of the Iranian medicinal plants, root extract of *Zhumeria mjadaei* showed strong antileishmanial activity. The extract was subjected to bioassay guided fractionation to purify biological active compound 1 using VLC and Centrifugal Preparative Thin Layer Chromatography (CPTLC). The potent *in vitro* activity of 12,1 dideoxy aegyptinone B 1 against *L. donovani* promastigotes (IC_{50} = 0.75 µg/ml) in the absence of obvious cytotoxicity on mouse macrophages (IC_{50} = 13 µg/ml) and monkey kidney fibroblast (IC_{50} = 12 µg/ml) formed the basis for further study of this promising lead activity. Moderate cytotoxicity on different kind of cancer cell lines including SK-MEL, KB, BT-549, SK-OV3 were observed. This compound was not so much active on *Plasmodium falciparum*.



Bactericidal and fungicidal activities of seeds from *Calia secundiflora* (Ort.) Yakovlev

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Calia secundiflora (Ortega) Yakovlev [syn. *Sophora secundiflora* (Ortega) Lag. ex DC] is a woody member of the Fabaceae distributed in Africa, America and Asia.[1] On the American continent its range extends from the southwest United States to the mountains of Oaxaca and Puebla in southern Mexico. It is considered a medicinal plant in Mexico but has scarcely been used due to its toxicity.[2] The purpose of this study was to identify the alkaloids in the seeds and evaluate the bactericidal and fungicidal activities of organic extract and a reference compound. Alkaloids were identified by Liquid chromatography-mass spectrometry (LC-MS): lupinine, anagyrine, sparteine, *N*-methylcytisine, 5,6-dehydrolupanine, and lupanine, the most abundant was cytisine.[3] *In vitro* bioassay was done with three species of phytopathogens fungus *Alternaria solani, Fusarium oxysporium* and *Monilia fructicola,* and three species of bacteria *Pseudomonas* sp, *Xanthomonas campestris* and *Erwinia carotova.* Comparative analysis of the Tukey media test were made. A slightly different tendency was observed; the statistical analysis demonstrated the absence of significant differences between the dichloromethane extract and the alkaloid with respect to the fungicidal activity. It was observed that the crude seed extract of *Calia secundiflora* is moderately active as fungicide and more potent as bactericide. In contrast the reference compound (cytisine) showed the opposite effects.

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Phytochemical constituents of fresh and dry leaves of *Gongronema latifolium*

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Gongronema latifolium, a leafy vegetable abundant in the tropical forests of Southwestern Nigeria and some parts of West Africa is used as a spice for sauces and as a medicinal plant. There are literature reports of its use in the treatment of diabetes, dysentery and antiparasitic properties. The use of medicinal plants in traditional healing is holistic, therefore, it is important that systematic studies will be undertaken to identify the bioactive components of *Gongronema latifolium* responsible for its healing properties.

Methanol extracts of fresh and dried samples were fractionated in butanol, chloroform, hexane, ethyl acetate and water to identify the active constituents. We report the following compounds being present in the crude methanol and fractionated extracts of *Gongronema latifolium*- alkaloids, flavonoids, cardiac glycosides, free and bound anthraquinones, tannins, and saponins. No cyanide was detected. The results of TLC and HPLC are presented.

The power of alliances: multiple induced chemical defenses protect norway spruce against a bark beetle species and its symbiotic fungus

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The terpenoid resin and phenolic constituents of conifers have been implicated in protecting trees against bark beetles and other enemies, but it has been difficult to prove these defensive roles under natural conditions. In the present investigation, we used methyl jasmonate (MJ), a well-known inducer of plant defense responses, to manipulate the biochemistry and anatomy of mature *Picea abies* trees and test their resistance to attack by *Ips typographus* and its fungal associate *Ceratocystis polonica*. Methyl jasmonate treatment induced the formation of traumatic resin ducts in the developing xylem, enhanced resin flow, and stimulated increased accumulation of monoterpenes, sesquiterpenes, and diterpene resin acids. However, only minor changes were detected in terpene composition, and no changes were measured in soluble phenolic content. The observed chemical and anatomical changes were correlated with increased resistance to *Ips typographus* and its fungal associate *Ceratocystis polonica*. Bark sections of *P. abies* treated with MJ had significantly less *I. typographus* colonization than control bark sections and exhibited shorter parental galleries and fewer eggs deposited. The growth of the blue-staining fungus *C. polonica* into the sapwood and the necrosis of the cambium caused by fungal invasion were both significantly reduced by methyl jasmonate application. The increased amount of terpenoid resin present in MJ-treated bark could be directly responsible for the increased resistance observed against *I. typographus* and *C. polonica*

Artemisinin and precursor compounds from *Artemisia annua* grown in South Italy

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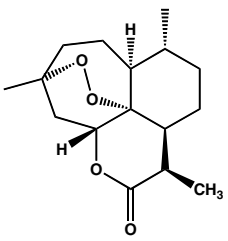
Artemisia annua ("qinghao"), sweet wormwood, is an annual herb native to Asia, now worldwide naturalized, including Italy. In traditional medicine the plant has been used for century against fever and in the treatment of malaria. The active compound, artemisinin, is a sesquiterpene lactone synthesized in the aerial organs of the plant; starting from this molecule several important semisyntetic antimalarial analogues have been derived.

The biosynthetic process to form artemisinin has been studied but still not fully understood. From a bisabolol-type precursor, through the intermediate amorpha-4,11-diene, *via* oxidation and reduction reactions, artemisinic and dihydroartemisinic acid are formed. This last metabolite may be chemically converted into the active principle. Artemisinin may also originate from arteannuin B. Higher levels of artemisinin in the plant are desirable for commercial applications, therefore field crop experiments to improve *A. annua* production and the yield of the active compounds can be undertaken. Moreover, the knowledge of the exact biosynthesis and/or the isolation of artemisinin precursors may allow the application of biotechnological methods.

The aim of our study was to evaluate the metabolic productivity (artemisinin and related metabolites) of *A. annua* grown in South Italy. Chromatographic purification of dichlorometane extracts of *A. annua* dried leaves allowed us to isolate artemisinin (**1**), deoxyartemisinin (**2**), arteannuin B (**3**) and dihydroartemisinic acid (**4**). Chemical characterization of these metabolites was achieved by ESI-MS and NMR. The metabolic profiling indicated that *A. annua* grown in South Italy has the potential to become a crop for pharmaceutical applications.



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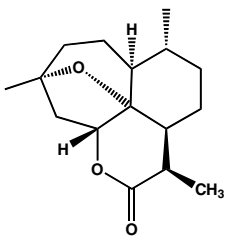

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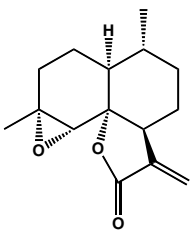

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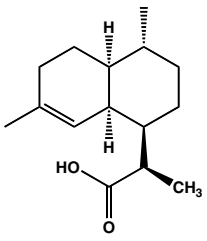

4

Sage bioactive compounds: phytochemistry, bioactivity and biotechnology aspects

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Sage (*Salvia officinalis* L.) enjoys the reputation of a panacea given its wide range of uses. The compounds responsible for sage medicinal properties are constituents of its essential oil, or alcoholic extract or *n*-hexane extract. This one, obtained in Soxhlet, gather all the essential oil compounds and phenolic constituents, namely phenolic diterpenes, besides the lipid-like compounds. However, sage is mostly used popularly as leaves infusion. In this context we have studied the respective composition, identifying most of their constituents, as well as their putative hepatoprotective/hepatotoxic, hypoglycaemic, and antigenotoxic activities. The sage infusion contained less than an half the essential oil constituents, phenolic acids and flavonoids. The drinking of sage infusion resulted in an improvement of the liver antioxidant status in rat and mice as well as their hepatocytes, as determined by in *in vivo* and *in vitro* tests by quantification of plasma transaminase, GST and GR enzyme activities. We have also shown that the water and methanolic sage extracts, as well as some of their phenolic compounds, namely luteolin and quercetin, protect the hepatocellular carcinoma cell line, HepG2, from *t*-BHP induced oxidative damage, although the sage infusion increases the CCl4-induced hepatotoxicity in mice. On the other hand, the metformin-like effect induced in rats supplied with sage infusion suggests that sage can be used in the prevention of type 2 diabetes mellitus. In parallel, the production of some of the bioactive compounds in sage *in vitro* systems, as shoots and suspended cells, has been followed envisaging their biotechnological production. Shoots have been the best system for production of essential oil constituents and cells suspensions the best system for production of phenolic acids and phenolic diterpenes. The accumulation profile of each phenolic compound over the growth cycle varies greatly allowing different phenolic mixtures in a narrow gap of time.

This work was sponsored by the FEDER and Portuguese Government through the Project FCT-POCI/AGR/62040/2004.

Human berry intervention study – microbial metabolites of berry phenolics from faecal samples

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Controlled human intervention was carried out to explore the effects of diet rich in berries on subjects with metabolic syndrome. The subjects were randomly assigned in three diet groups: A) ellagitannin rich berries (cloudberry, strawberry, raspberry), B) anthocyanin rich berries (bilberry), and C) control. During the diet faeces samples were taken at five occasions: 1) four weeks before berry intervention, 2) one week before berry intervention, 3) when berries had been consumed for four weeks, 4) when berries had been consumed for seven weeks, and 5) four weeks after the berry intervention ended. The faeces were closed and stored in anaerobic atmosphere, cooled, and frozen at –70 °C in 4 h after defecation. For analysis faeces samples were melted slowly in ice – water bath, and metabolite analysis was carried out from 5% faecal suspension in physiological saline by GC-MS using selective ion mode (SIM) detection.

Large amounts of phenolic compounds are likely to enter the colon, where they are metabolised by gut microflora. There is very little information of the bacterial transformations of some groups of phenolic compounds, such as anthocyanins. In order to increase knowledge of the metabolism of phenolic compounds, and the role of gut microflora in the overall metabolism, targeted analysis of the known faecal metabolites of phenolic compounds were carried out. The following 11 compounds were analyzed from faecal samples: 3-hydroxyphenylacetic acid (3-OHPAc), 3-phenylpropionic acid (3-PPr), benzoic acid (BA), 3,4-dihydroxybenzoic acids, 3,4-dihydroxyphenylacetic acid (3,4-diOHPAc), 3,4-dihydroxyphenylpropionic acids (3,4-diOHPPr), 2-hydroxyphenylpropionic acid (2-OHPPr), 3-hydroxyphenylpropionic acid (3-OHPPr), 4-hydroxyphenylpropionic acid (4-OHPPr), 3-hydroxybenzoic acid (3-OHBA) and 4-hydroxybenzoic acid (4-OHBA). Concentrations of these compounds were followed as a course of time of intervention. Statistical evaluation of the results was performed using paired Student's t-test. The comparison showed that ellagitannin rich diet significantly increased concentration of 3,4-dihydroxybenzoic acid (3,4-diOHBA) ($p < 0{,}001$) and 3-hydroxybenzoic acid (3-OHBA) ($p < 0{,}001$) compared to period before berry diet. Anthocyanin rich diet significantly increased concentration of 3,4-dihydroxybenzoic acid (3,4-diOHBA) ($p < 0{,}001$) and 3-hydroxyphenylacetic acid (3-OHPAc) ($p < 0{,}01$). The results verified the earlier *in vitro* data of the metabolism of phenolic compounds.

PLANT CELL AND TISSUE CULTURE
C1–C12

Arctic bramble cell cultures as a source of berry phenolics

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Berries are rich in phenolic compounds with beneficial biological properties for human health. Arctic bramble (*Rubus arcticus*) grows in borealic zone, and its ruby red berries are well known for good taste and flavour. In addition, arctic bramble is rich in bioactive phenolic compounds, especially the complex phenolic polymer ellagitannin, which is reported to have e.g. *in vitro* anti-oxidant activities and antimicrobial effects against human pathogens. The colour of arctic bramble originates from anthocyanins, which also possess variable beneficial effects on human health. The crop of both wild and cultivated arctic bramble is very low, and therefore cell cultures are the potential choice for production of already characterized and novel secondary metabolites for food and pharmaceutical purposes.

Arctic bramble cell cultures were established from cuts of sterile *in vitro* leaves on medium with plant hormones kinetin and NAA (α-Naphtalen-acetic acid). Callus lines were grown on the hormone medium, and good growth and bright colours were used for selection criteria for maintenance and initiation of suspension cultures. Phenolic compounds were measured from stable callus and suspension cultures obtained, and selected culture was used for elicitation experiment aiming to increase production of phenolic secondary metabolites. Elicitors used were methyl jasmonate, ethephon, S-ABA and chitosan, and they were introduced in the cultures in late logarithmic growth phase. Samples collected at different time points were filtered, and the cell mass was freeze-dried and extracted with methanol. Freeze-dried extracts were analysed by HPLC-DAD.

In callus and suspension cultures phenolic acids were the main phenolics detected in methanol extracts. Elicitation clearly enhanced the production of some phenolic compounds in suspension cultures, and elicitation with S-ABA also promoted synthesis of a new phenolic compound, which still needs to be characterized.

In vitro techniques and genetic engineering of *Veratrum californicum* Duran

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The genus *Veratrum* is distributed throughout the northern temperate and Arctic regions of Europe, Asia and North America. *Veratrum californicum* Duran (Melanthiaceae) is an important monocotyledonous medicinal plant which is the only source of the anticancer compound cyclopamine. In order to be able to utilize *Veratrum* cells in the production of important secondary metabolites, an *in vitro* platform is needed. For that purpose, tissue culture, green plant regeneration and genetic engineering of *Veratrum californicum* were developed. Fine suspension cell lines were established from germinated mature embryos by transferring friable embryogenic calli to AA- and L2-medium. The suspension cells were cryopreserved successfully and recovered at a high rate. In addition, a highly embryogenic and regenerating cell line was established and kept on L2-medium without additional growth regulators. Very good growth was obtained by employing the temporary immersion system of RITA®-bioreactors. The highly regenerating cell line and *in vitro* plantlets contained steroid alkaloids cyclopamine and veratramine. Furthermore, transformation methods were developed for *Veratrum californicum*. Transgenic cell lines were produced by *Agrobacterium tumefaciens* and *A. rhizogenes* -mediated deliveries. Also direct gene transfer techniques such as particle bombardment and protoplast-based techniques were applied. The basic tools for the metabolic engineering and biotechnological production of secondary metabolites of *Veratrum californicum* are now available.

Alkaloid production in stressed root cultures of *Uncaria tomentosa*

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Uncaria tomentosa (cat's claw), an indigenous plant from the Amazon rainforest, is the source of pentacyclic monoterpenoid oxindole alkaloids (PMOA) with immunomodulatory, cytotoxic, anti-AIDS and antileukemic activities. PMOA are obtained from powered bark of more than 8-years old native plants and their synthesis is very complex. These highly oxidized alkaloids have been recently produced in cell suspension cultures of *U. tomentosa* but in low concentrations.[1] In an attempt to increase PMOA production, fast-growing and hormone independent root cultures were established. Root cultures showed, by reverse phase HPLC, accumulation of PMOA (0.8–1.2 mg/g DW), mainly mitraphylline, isomitraphylline, pteropodine and isopteropodine. Also, the cultures produced a pentacyclic glucoindole alkaloid, which was not previously detected in the wild plant. The structure of this compound with hypotensive activity was verified by mass spectrometry and 1D and 2D NMR spectroscopy as 3α-dihydrocadambine (DHC). The effect of some stress conditions as feeding with high concentration of sucrose, jasmonic acid (JA) elicitation, or addition of an oxidant agent (buthionine sulfoximine, BSO) on PMOA was investigated. As a result of osmotic stress with 7% sucrose or the oxidative condition (0.8 mM BSO), PMOA production was 3–3.5 or two-fold higher than the non-stressed cultures, respectively. In contrast, JA (200 μM) did not stimulate PMOA production while DHC production was three-fold stimulated. A combination of JA elicitation and sucrose feeding increased the DHC production to 1 mg/gDW which was five-fold higher than that of non-elicited cultures.

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1. G. Trejo-Tapia *et al.*; Monoterpenoid oxindole alkaloid production by *Uncaria tomentosa* (Willd) D.C. cell suspension cultures in a stirred tank bioreactor. *Biotechnol. Prog.* 2005, 786–792.

Biosynthesis of oxindole alkaloids in plantlets and roots cultures of *Uncaria tomentosa*

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Alkaloids are defense-related metabolites that are accumulated in specific cells and tissues during plant development. *Uncaria tomentosa* (cat's claw) a native plant from the Amazon rainforest produces pentacyclic monoterpenoid oxindole alkaloids (PMOA) which possess immunomodulatory, cytotoxic, antileukemic and anti-AIDS properties. In order to understand the regulation of PMOA biosynthesis in *U. tomentosa*, we fed jasmonic acid-elicited and non-elicited *in vitro* plants and root cultures with PMOA potential radiolabeled precursors and analyzed the accumulated products. In the aerial vegetative organs (young and old) of plantlets supplied with ^{14}C-tryptophan (100 μM), radioactive tryptamine and the highly oxidized PMOA rapidly accumulated. In root cultures, the radioactivity was incorporated into PMOA and into a glucoindole alkaloid, which was not previously detected in the wild plant. The structure of this compound was verified by mass spectrometry and 1D and 2D NMR spectroscopy as 3α-dihydrocadambine (DHC). Addition of the terpenoid precursor loganin or jasmonic acid increased the flux towards secologanin, indole alkaloids and DHC in roots, while clomazone, the terpenoid enzyme inhibitor of deoxyxylulose synthase inhibited alkaloid production. We also have evidence that DHC acts as intermediate in PMOA biosynthesis in cultured roots while in leaves a direct transformation of the glucoindole alkaloid to PMOA takes place. These findings and our recent progress in the isolation of the oxidative enzymes involved in the transformation of monoterpenoid indole alkaloids to PMOA will be discussed.

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Metabolism of phenylpropanoids in grapevine cell cultures

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Polyphenols, including flavonoids and stilbenes, are an essential part of human diet and constitute one of the most abundant and ubiquitous groups of plant secondary metabolites. The level of these compounds is inducible by several abiotic and biotic (e.g. fungal attack) stresses, so attempts are being made to identify elicitors. Despite their limited occurrence in nature, stilbenes have recently received much attention due to their numerous biological activities. Resveratrol is in fact believed to be a major contributor to the health benefits associated with the moderate consumption of red wine (the so called "French paradox"), having antioxidant, anti-inflammatory and anticancer activities.

We investigated the activation and the regulation of the phenylpropanoid biosynthetic pathway in *Vitis vinifera* (cv. Barbera) cell cultures supplied with different elicitors, such as chitosan, *N*-acetyl-D-glucosamine, D-glucosamine, rifampicin, ampicillin, methyl-jasmonate and jasmonic acid. During a 15 days time-course, cell growth parameters, total anthocyanin amount, endogenous and released levels (HPLC-DAD) of stilbenes (resveratrol and its mono-glucosides), hydroxycinnamic acids, catechins and other flavonoids, were monitored. Methyljasmonate and chitosan seemed to be the most effective elicitors. Proteomic analyses were performed on methyl-jasmonate and chitosan treated cells, showing that both elicitors up-regulated the expression of stilbene synthase (STS), the resveratrol-forming enzyme. The expression of several other proteins was specifically affected by the treatments (e.g. PR-10). Northern blot mRNA expression analyses of the main enzymes involved in the phenylpropanoid biosynthetic pathway (e.g. PAL, CHS, CHI, STS), were also performed.

An integrated technology consisting of biomass production (callus and liquid cell cultures), bioreactor fermentation of cell suspensions, extraction and purification of the bioactive polyphenols, is at moment being developed.

Production of shikonin and derivatives via callus and suspension culture of *Arnebia densiflora L.*

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A perennial species *Arnebia densiflora* (Nordm.) Ledeb. is known as endemic to Turkey and Greece. Root, mostly root cortex of the plant contains higher levels of alkannin/shikonin derivatives than the other species of Boraginaceae family. *A. densiflora* has been utilized for its wound healing properties by human in some of the Mediterannean countries for ages. So far, a number of biological activities including anti-cancer, anti-inflammatory, anti-microbial, anti-oxidant, anti-HCV (against hepatitis-C), and anti-HIV activities have been observed for the extracts of the plant. Shikonin derivartives have also been used in dermatological preparation, in cosmetics, and in food/textile industry as a colorant. The aim of our study is to produce callus from different parts of *A. densiflora* by employing tissue culture methods, which has never been attempted before. Different media formulations, LS, MS and SH, were applied supplemented with Kinetin, IAA, 2,4-D and NAA to produce callus cultures. According to our observations, fresh roots and flower axis formed better callus than the other parts of the plant. From pink to red callus were observed. Cell suspension culture is being established.

Production and increase of cucurbitacin B in *Ecballium elaterium* (L.) A. Rich. cell suspension culture by using elicitors

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Ecballium elaterium has been widely used in treatment of sinusitis as a folk medicine in Turkey. Cucurbitacin B is isolated as an active principle that has antiinflammatory effect. The goal of this study is to increase cucurbitacins, specifically cucurbitacin B content in *E. elaterium* callus and cell suspension cultures by using plant tissue culture techniques. Callus cultures were established from the stem nodes, leaves and fruits on MS (Murashige and Scoog) medium supplemented with different growth regulators. Medium I consists of 1 mg^{-1} benzyladenin (BA), 0.5 mg^{-1} naphtalenacetic acid (NAA), Medium II consists of 1 mg^{-1} BA, 0.1 mg^{-1} NAA. Cucurbitacin B was determined in callus cultures, whereas suspension cultures are under the investigation. Cucurbitacin B will be analyzed in suspension cultures, especially in fruit originated callus and suspension cultures. A variety of elicitors will be applied to increase the Cucurbitacin B levels in suspension cultures as a first step for the large scale production.

Induction of secondary metabolite diversity with phytohormones and methyl jasmonate treatment in *Psoralea drupaceae in vitro* cell cultures

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Genus *Psoralea* is known in Chinese medicine as source of wide range of phenolic compounds with antibacterial, antitumour, anti-inflammatory and antipyretic activities, among them furanocumarin psoralen and phenolic meroterpene bakuchiol are the best studied. Up to now only few reports have been published concerning phytochemical constituents of *in vitro* cell cultures of *Psoralea* species. In our work we established for the first time cultures of dedifferentiated cells of *Psoralea drupaceae* and studied patterns of secondary metabolites in the cells under various combinations of phytohormone and methyl jasmonate treatments.

The callus cultures of *Psoralea drupaceae* were established and grown on MS medium containing 2 mg/L of 2,4-D, 1 mg/L of NAA, 0.1 mg/L of BAP and 0.1 mg/L of kinetin. After several passages cultures were grown on the MS media supplemented with 16 different combinations of auxins and cytokinins and, additionally, elicited with methyl jasmonate. The cells were harvested, extracted and the extracts were monitored by reversed-phase HPLC and tested for antibacterial activity.

As a result we observed considerable diversity of the secondary compound patterns in the tested extracts. Bakuchiol, which was identified on the basis of HPLC data and comparison with the standard, was detected in 5 of 16 variants of phytohormone treatments. Concentrations of several still unidentified compounds increased up to 10-fold after methyl jasmonate elicitation in selected phytohormone variants. Thus we can state that combined influence of phytohormone and jasmonate strongly increases biodiversity of secondary compounds in *Psoralea drupaceae in vitro* cell cultures.

Differential induction by methyl jasmonate of tocopherol content and tyrosine aminotransferase activity in *Amaranthus caudatus* and *Chenopodium quinoa* cultured cells

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Tocopherols and tocotrienols (collectively known as vitamin E) are isoprenoid compounds, which consist of a hydrophilic chromanol head and a hydrophobic isoprenoid tail. Depending on the degree and position of methylation of the chromanol ring, there are four forms of tocopherols (δ-, β-, γ-, α-tocopherol). They are synthesized and accumulated in all green tissues of photosynthetic organisms, even though significant amounts are frequently observed in seeds. In plants, tocopherols are believed to protect chloroplast membranes from photooxidation and help to provide an optimal environment for the photosynthetic apparatus, thus possessing an antioxidant role. Besides these functions in plant metabolism, tocopherols are essential components of the human diet. α-tocopherol deficiency leads to a number of disorders and there is strong evidence that vitamin E supplementation is beneficial to health.

Plant cell cultures represent a possible method for the production of valuable plant compounds, and for a better understanding of plant metabolic pathways. Unfortunately, for many of the compounds of interest, yield is too low or even zero in cultured cells, and consequently other approaches, such as induction of pathways with various elicitors, have been used.

In this work, callus cultures were established from *Amaranthus caudatus* and *Chenopodium quinoa*, two species that are naturally rich in tocopherols, with the aim of verifying if callus cultures maintain the capacity of producing these compounds. Since a close relationship between jasmonic acid and tocopherol levels in plants has been proposed, and in *Arabidopsis* jasmonates can induce tocopherol biosynthesis via induction of tyrosine aminotransferase (TAT) activity, one of the key enzymes in the biosynthetic pathway of these compounds, the effect of methyl jasmonate (MJ) on *in vitro* cultures of these two plants was also evaluated. Results show that, in vivo, α-tocopherol is the main form present in leaves, while γ-tocopherol is found only in seeds. Callus cultures from both species produce very low levels of tocopherols, the α-isoform being the only one detected. In response to elicitation with MJ, tocopherol production and TAT activity were increased in *A. caudatus* cells, and the hormonal composition of the culture medium influenced the response. Conversely, no effect by MJ was observed in *Chenopodium* callus cultures. Thus, the differential modulation of α-tocopherol levels in these two plant cell cultures can provide a useful tool for investigating the regulatory mechanisms involved in this metabolic pathway.

Plant secondary metabolites production: Hydroponic cultures for harvesting root phytochemicals

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A new technology, based on hydroponic culture, was developed for the production of natural molecules having pharmaceutical and cosmetic interest. The present study relates to the production of two tropane alkaloids (hyoscyamine and scopolamine) from *Datura innoxia* Mill. cultivated under different conditions. A comparison between permanent and temporary immersion of *D. innoxia* roots during hydroponic culture was performed. In the temporary immersion system the plants grew faster (growth improved by 89%) and secondary metabolite levels were more than doubled. Addition of *Agrobacterium rhizogenes* (ATCC TR7) into the nutrient solution of hydroponic system was also tested. After 32 days of culture, the alkaloid levels were multiplied by 2.3 in the *Agrobacterium* treated plants (dry matter production: +72%). Hydropony in temporary immersion system is a simple and efficient method that leads to improved plant biomass and secondary metabolites productivity. *A. rhizogenes*, can also stimulate plant growth and alkaloid synthesis in such hydroponic conditions.

Disposable bag bioreactor for plant cell and tissue cultures

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The superiority of low-cost and disposable bioreactors with a gas-permeable cultivation bag of plastic film was effectively proven in a number of plant cell cultivations. The single-use cultivation bags are partially filled with medium, inoculated with cells, and discarded after harvest. This makes cleaning and sterilization in place unnecessary and guarantees high flexibility as well as process security with contamination levels below 1%. The BioWave reactor being the first mechanically driven, scalable bag reactor has a leading position among disposable bioreactors. Due to the rocking movement of the platform the surface of the medium is continuously renewed and bubble free surface aeration takes place. In the BioWave we found that the modified Reynolds number, the mixing time, the residence time distribution, the oxygen transfer efficiency and the specific power input is dependent of the rocking angle, the rocking rate, the culture bag type (CultiBag) and its geometry, as well as the filling level. Mixing times between 10 and 1400 s were determined. Experiments which focused on residence time distribution have demonstrated that a continuously operating BioWave in perfusion mode can be described by the ideally mixed stirred tank model. Oxygen transfer coefficients achieved in the BioWave reached comparable or even higher values than those which have been reported for stirred, bubble-free aerated or surface aerated bioreactors.

Moreover, our studies reveal the potential of the BioWave for cultivating tobacco, grape, apple and yew suspension cell cultures as well as hairy root cultures of devil's claw, Egyptian henbane and Asian ginseng. We worked with culture volumes from 0.4 to 10 L (suspension cultures) and 0.5 to 5 L (hairy root cultures). For secondary metabolite-producing or protein-expressing plant suspension cells, we achieved maximum biomass productivities of 40 g fw L^{-1} d^{-1} and excellent doubling times of 2 days. Finally, the paclitaxel productivity accomplished in BioWave with immobilized *Taxus* suspension cells is one of the highest reported so far by academic researchers for *Taxus* species cultures in bioreactors. Encouraging results were also obtained for hairy roots cultivated in ebb-and-flow mode. We regularly achieved biomass productivities and product yields of specific hairy root clones in the BioWave operating with a 2 L CultiBag specific which were two to three times higher than in tested spray reactors.

Antioxidant dietary supplements from *in vitro* Labiatea cell lines

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The project NUTRASNACK (E.C. F.P.6 contract No FOOD-CT-2005-023044) has the main objective to increase the antioxidant potential of food snacks in collaboration with ENERVIT®, a well-known firm in the development of nutrition programs to enhance the performances of athletes and sportsmen. Several plant species of the *Labiatae* family have been tested for the presence of specific antioxidant metabolites and for their total antioxidant capability. The genotypes with the highest antioxidant values were selected within each species. The aseptic cultures of specific genotypes such as *Salvia spp., Ocimum spp.,* and *Mentha spp.* were established *in vitro*. The suitable protocols for the development of safe and friable calli were studied and the use of plant growth regulators without human health risk was evaluated. Futhermore the friable calli were disgregated in the liquid medium and the corrispondent cell cultures were established to enhance the biomass development. The growth parameters such as viability, PCV, growth curve in dry and fresh weight gave the essential information for the management of the cultures. The antioxidant total capacity was evaluated during all the growth phases either with FRAP, and DPPH tests. These prelimary results were compared with the content of the most aboundant antioxidant secondary metabolites such as rosmarinic acid, carnosic acid, carnosol and flavonoids. The plant material samples were extracted by maceration with EtOH-W (7:3) and analysed by LC-DAD-ESI-MS in order to evaluate their chemical composition. All these experimental studies represented the preliminary studies for the establishment of an authomatized system of cell biomass culture which was able to guarantee the low cost production of specific natural antioxidants.

ENGINEERING OF BIOSYNTHETIC PATHWAYS
D1–D22

Molecular cloning, functional expression and characterization of four sesquiterpene synthases from *Cistus creticus* ssp. *creticus*

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Cistus creticus ssp. *creticus* is a member of the Mediterranean flora, widely known for the characteristic resin "ladanon", used since ancient times for its aromatic and pharmacological properties. The resin, produced by the *Cistus* glandular trichomes, consists mainly of terpenoids and flavonoids. Previously, a bioinformatic analysis of an EST database from *Cistus creticus* ssp. *creticus* trichomes identified four cDNA clones with homology to sesquiterpene synthases. Here, we report the isolation of the four full-length cDNAs, named *CcTPS1-4* and the biochemical characterization of the proteins they encode. The four cDNAs contain 1656–1752 bp open reading frames encoding proteins of 551–583 amino acids (64.9–65.5 kD). The similarity of their deduced amino acid sequences ranges from 55% to 77%. The enzymes they encode use farnesyl diphosphate as a substrate and catalyze the formation of multiple products. The enzymes were named after their major product and corresponded to β-caryophyllene synthase (CcTPS1), β-farnesene synthase (CcTPS2), germacrene B synthase (CcTPS3) and E-nerolidol synthase (CcTPS4). Phylogenetic analysis, based on protein similarities, clusters CcTPS1, CcTPS3, CcTPS4 together and CcTPS2 as outgroup, indicating different evolutionary events for the two groups.

Suppressed ascorbate oxidase activity increased ascorbate redox state, arrested fruit growth and altered fruit ripening characteristics in melon transgenic lines harbouring a gene coding for ascorbate oxidase

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L-Ascorbic Acid (AA, Vitamin C) is an essential nutrient that vertebrates have lost the ability to synthesize and therefore they depend on dietary sources to meet needs. AA plays important roles in metabolism acting as a cofactor for many enzymes and in scavenging free radicals. In plants, it is also a crucial compound, with important roles as an antioxidant, enzyme cofactor and as a donor/acceptor in electron transport. The last few years it has been proposed a regulatory role of AA in cell division and expansion, as well as in cell wall loosening. One of the enzymes responsible for the oxidation of AA is ascorbate oxidase (AO, EC 1.10.3.3). Ascorbate oxidase belongs to the copper-containing enzyme family and catalyzes the oxidation of AA to dehydroascorbic Acid (DHA) via monodehydroascorbic acid (MDHA). Although various biochemical functions have been proposed for AO, its precise physiological role remains unclear. To explore the role of AO in fruit physiology, growth and nutrition, homozygous melon transgenic lines in which the expression of AO was suppressed through genetic engineering were evaluated in terms of growth, weight, firmness, ethylene and carbon dioxide production. The resultant large suppression of about 99% in AO activity in transgenic fruit caused a dramatic arrest in fruit growth as measured by weight starting from 32 days after pollination (DAP) until the completion of ripening at 40-DAP. Simultaneously, an increase in ethylene and carbon dioxide production were observed in ripe transgenic fruit which resulted in early ripening and a decrease in fruit firmness compared to wild type fruit. Further, this genetic modification increased the apoplastic AA and decreased the DHA, thus altering the ascorbate redox state in transgenic melon fruit. Further analysis of the melon transgenic lines at the molecular and biochemical levels will help us to understand the AO participation in fruit growth, cell wall expansion and cell wall softening.

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Modeling of plant secondary metabolism for development of plants with improved pathogen resistance

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The amino acid-derived glucosinolates and indole alkaloid phytoalexins are important natural plant compounds characteristic for cruciferous plants. Upon tissue disruption glucosinolates are hydrolyzed by myrosinases to produce degradation products, typically isothiocyanates, thiocyanates, and nitriles, involved in plant defence. Indole alkaloids are induced in cruciferous plants upon pest attack and play a role in defence against specific fungi. Recent progress in the understanding of the biosynthesis of both indole phytoalexins and glucosinolates has revealed the central role of the cytochromes P450 of the CYP79 family. Very little is known about the biosynthesis of *Brassica spp.* indole alkaloids, and knowledge gained with camalexin in *Arabidopsis thaliana* is expected to be transferable to *Brassica* metabolites. The objective of this project is to determine the role of natural products, especially glucosinolates and indole phytoalexins in conferring resistance to different pests. The aim is to develop novel strategies for pest management in *Brassica* crops to reduce chemical input in the form of pesticides and provide an important step towards attaining a durable and sustainable agriculture. Oilseed rape (*Brassica napus*) is the number one oil crop of the Nordic countries. The beneficial fatty acid composition of the seed oil for human consumption and applications of modified seed oil for biofuels and technical use indicate that this crop will increase in use locally and worldwide.

Rosmarinic acid synthase – a new tool to engineer rosmarinic acid biosynthesis

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Rosmarinic acid synthase (RAS; hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyltransferase) is the central enzyme of rosmarinic acid (RA) biosynthesis, transfering the hydroxycinnamoyl moiety of a hydroxycinnamoyl-CoA to the aliphatic hydroxyl group of a hydroxyphenyllactate. RA has been an interesting compound for pharmaceutical, cosmetic and food industry due to its antiviral, antibacterial, antioxidative and antiinflammatory properties. Until now, RA is isolated from plants since a biotechnological production with plant cell cultures was not economically feasible. Recently, a RAS-cDNA has been isolated from *Coleus blumei* (Lamiaceae)[1] opening up the possibility to engineer RA production in plants, bacteria or yeast. The RAS-cDNA sequence has an ORF of 1290 bp encoding a protein of 430 amino acid residues with a calculated molecular mass of 47902 Da. Comparison of cDNA- and genomic sequences revealed an intron of 914 bp inserted after 405 coding nucleotides. The RAS sequence shows high similarities to other hydroxycinnamoyltransferases, e.g. hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyl-transferase involved in the biosynthesis of caffeoylshikimate and chlorogenate via 4-coumaroylquinate or -shikimate, a step that has recently been identified to be essential for introducing the 3-OH group into phenylpropanoids. RAS, however, does not accept quinate and shikimate as acceptor substrates, thus showing its specificity for the biosynthesis of RA and RA-like esters. RAS belongs to the superfamily of BAHD acyltransferases[2,3] showing typical conserved sequence motifs such as the HxxxD(G)-motif in the catalytic center and the DFGWG motif. Overexpression of RAS in plants, bacteria or yeast are currently under way and might allow a higher production of RA for economic processes.

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Engineering the glucosinolate pathway into potato

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Glucosinolates are amino acid-derived secondary metabolites present in the agriculturally important *Brassica* vegetables, such as e.g. oilseed rape and broccoli. Their bioactivity ranges from functioning as feeding attractants/deterrents for herbivores to conferring specific pest resistance to crops. They are known to humans for their cancer-preventive properties, their characteristic mustard flavour, and their use as biopesticides. The synthesis of the core structure of glucosinolates involves at least 5 different gene products, probably assembled as a metabolon complex. With the recent identification of the *C-S* lyase, the *S*-glucosyltransferase, and the sulfotransferase, and the previously known cytochromes CYP79 and CYP83, transfer of the glucosinolate pathway to heterologous hosts has become a realistic goal. We have initiated efforts to engineer the whole pathway into potato, in order to confer resistance to the late blight disease and the brown rot. Since the traditional transgene stacking is not convenient (considering the number of genes involved), an alternative method has been adopted. This method utilizes virus-derived sequences as part of one-ORF multicistronic constructs, which allow the expression of several genes from a single promoter sequence.

Effects of transformation on the flavonoid pathway genes of the grapefruit plants

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In grapefruit, the flavanone glycosides are the major flavonoids, which are produced throughout the plant. They affect the taste rather than color. Flavanone rutinosides are tasteless, while flavanone neohesperidosides are bitter. Naringin is the major bitter flavanone glycoside in grapefruit, which deprives consumers having nutritional and health benefit of them. Main aim is to engineer the biosynthesis of flavanone glycosides in grapefruit using molecular genetics and transformation techniques in order to decrease the bitter taste or increase the flavonones for pharmacological and industrial value. cDNAs of the structural genes chalcone synthase (CHS) and chalcone isomerase (CHI) were isolated from citrus. Sense and antisense constructs of these cDNAs were transferred to grapefruit to suppress the target gene expression and/or increase the nonbitter flavonoid compounds, which may have pharmacological benefits and industrial value. Further, 1,2 rhamnosyl transferase7 (1,2 RT) cDNA, which catalyzes the last biochemical step in the production of naringin were utilized for the transformation. All the transfered gene constructs showed different effects on the plants. Transformed plants were analyzed for their transgene copy numbers via molecular techniques and flavonoid content by chromatographic methods. They are being evaluated for their morphology and steady state RNA levels.

A putative pinoresinol-lariciresinol reductase from *Phaleria macrocarpa* (Scheff.) Boerl.

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Phaleria macrocarpa (Scheff.) Boerl., a member of the Thymelaeaceae, is traditionally used in Indonesia as medicinal plant against cancer, diabetes, hypertension, and cardiovascular diseases. Within this study we investigate the lignan composition also with respect to the stereochemistry in this plant, since species of the Thymelaeaceae accumulate lignans with S-S configuration instead of the usually occurring R,R-configuration at C-atoms 8,8'. We isolated the lignans pinoresinol, lariciresinol, and matairesinol from different parts of this plant by using a modified extraction method of Wichers *et al.* (1). The identification of the lignans was conducted by using HPLC and LC-MS. According to chiral HPLC, pinoresinol and lariciresinol were mixtures of both enantiomers with 79 ± 3.5% e.e. and 55 ± 6.2% e.e. for the (-)-enantiomers, respectively, whereas matairesinol was found as pure (+)-enantiomer. Furthermore, a cDNA which probably encodes a pinoresinol–lariciresinol reductase (PLR) was cloned from this plant by a RT-PCR approach (2, 3), resulting in a full-length cDNA with 969 bp encoding a protein of 271 amino acids. This putative PLR shows highest similarities to the PLR of *Linum perenne* (67% identity and 80% similarity) and *L. usitatissimum* (65% identity and 78% similarity) on amino acid level. This is the first report on the occurrence of a pinoresinol–lariciresinol reductase in a species of the Thymelaeaceae.

Financial support by the DAAD is gratefully acknowledged.

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Salicylic acid accumulation in transgenic *Brassica rapa* transformed with a gene encoding bacterial isochorismate synthase gene

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The accumulation of salicylic acid (SA) in *Brassica rapa* ssp. *oleifera* transformed with a bacterial isochorismate synthase (ICS) gene was examined in the old and young leaves of the primary transformants through high performance liquid chromatography analysis. The level of SA and salicylic acid glycoside (SAG) in the transformed plants varied between the different lines but the SA contents of the leaves in all transgenic plants were increased significantly in comparison to the control plants. Increased SA levels were accompanied by increased accumulation of the SAG. This finding suggests that the ICS gene was expressed and functional in inducing SA biosynthesis in *B. rapa*. Higher level of SA was observed in young leaves than in old leaves of nine months old-plants whereas in eighteen months old plants old leaves showed highest levels of SA. The primary transformed plants showed a normal phenotype but the flowering plants produced less seeds.

Microarray analysis of putative target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*

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Genetic modification of the flavonoid pathway is an attractive target for metabolic engineering. It has been used to produce novel colors and color patterns in ornamental plants but also to modify nutritional and pharmaceutical properties of food crops. It has been suggested that coordinate control of multiple steps of the pathway with help of regulatory genes would lead to more predicted control of metabolic flux. Designing strategies for controlled metabolic engineering requires detailed characterization of candidate regulatory proteins and their target genes. We have studied regulation of anthocyanin biosynthesis in a common ornamental plant, *Gerbera hybrida* (Asteraceae). An R2R3-type MYB factor, GMYB10, shares high sequence homology and is phylogenetically grouped together with previously characterized regulators of anthocyanin pigmentation in *Petunia* and *Arabidopsis*. Ectopic expression of GMYB10 alone leads to strongly enhanced accumulation of athocyanin pigment levels as well as to altered pigmentation pattern in transgenic gerbera plants. Anthocyanin analysis indicates that GMYB10 specifically induces cyanidin biosynthesis in undifferentiated callus tissues, leaves and in normally acyanic floral organs. Furthermore, in flower petals enhanced pelargonidin production is detected. We have utilized the gerbera 9K cDNA microarray and made a comparison of gene expression profiles in transgenic and non-transformed tissues. Microarray analysis revealed several putative target genes for GMYB10 including new gene family members of the biosynthetic genes of the flavonoid pathway as well as new regulatory genes.

Cytosolic HPPK/DHPS from *Arabidopsis thaliana*: A specific role in early development and stress response

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In plants, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase (mitHPPK/DHPS) is a bifunctional mitochondrial enzyme, which catalyses the first two consecutive steps of tetrahydrofolate biosynthesis. Mining the *Arabidopsis* genome database has revealed a second gene encoding a protein that lacks a potential transit peptide, suggesting a cytosolic localization of the isoenzyme (cytHPPK/DHPS). When the N-terminal part of the cytHPPK/DHPS was fused to GFP, the fusion protein appeared only in the cytosol, confirming the above prediction. Functionality of cytHPPK/DHPS was addressed by two parallel approaches: first, the cytHPPK/DHPS was able to rescue yeast mutants lacking corresponding activities; second, recombinant cytHPPK/DHPS expressed and purified from *E. coli* displayed both HPPK and DHPS activities *in vitro*. In contrast to mitHPPK/DHPS, which was ubiquitously expressed, the cytHPPK/DHPS gene was exclusively expressed in reproductive tissue, more precisely in developing seeds as revealed by histochemical analysis of a transgenic cytHPPK/DHPS promoter-GUS line. In addition, it was observed that expression of cytHPPK/DHPS mRNA was induced by salt stress, suggesting a potential role of the enzyme in stress response. This was supported by the phenotype of a T-DNA insertion mutant in the cytHPPK/DHPS gene, resulting in lower germination rates as compared to the wild type upon application of oxidative and osmotic stress.

Sucrose-induced phosphorylation changes in plasma membrane proteins of *Arabidopsis*

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Sucrose is the main product of photosynthesis and the most common transport form of carbon in plants. In addition, sucrose is also a compound that serves as a signal affecting metabolic flux and development in plants. Here we present the first analysis of sucrose-induced phosphorylation changes of plasma membrane proteins in *Arabidopsis*. In an unbiased approach, seedlings were grown in liquid media with 30 mM sucrose for 7 days, then depleted for carbon in media without sucrose for 24-h before 30 mM sucrose was added, followed by immediate purification of plasma membranes, enrichment of phosphopeptides and subsequent analysis by mass spectrometry. In total, 67 phosphopeptides were identified, of which 60% were quantified over five time points of sucrose resupply. Among the identified phosphorylation sites, the well-described phosphorylation site at the C-terminus of plasma membrane ATPases shows a relative increase in phosphorylation level in response to sucrose. A new phosphorylation site was identified in the ATPase AHA1 and/or AHA2 and this phosphorylation site was shown to be crucial for ATPase activity and able to override regulation via the well-known C-terminal phosphorylation site. Novel phosphorylation sites were identified for both receptor kinases and cytosolic kinases which show rapid increases in relative intensities after short times of sucrose treatment. Seven response classes were identified including non-responsive, rapid-increase (within 3 min), slow-increase, and rapid-decrease. Relative quantification of phosphorylation changes by phosphoproteomics provides a means for unraveling fast signaling responses to external stimuli in plants and a basis for further functional characterization.

Vitamin bio-fortification of cereals for food security

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Vitamins play a vital role in human health. Deficiency of most vitamins can impair the immune system. Carotenoids are colored pigments found in plants that are precursors of vitamin A in human body. Vitamin A deficiency (VAD) is an immunodeficiency disorder characterized by widespread alterations in immunity. VAD is a major cause of premature death in developing nations, particularly among childern. About 800,000 deaths in children and women of reproductive age are attributable to vitamin A deficiency, which along with the direct effects on eye disease account for 1.8% of global disability-adjusted life years.[1] Vitamin E deficiency depresses immunoglobulin response to antigens, lymphocytic proliferative responses to mitogens and antigens, delayed dermal hypersensitivity reactions, and general host resistance. Vitamin C deficiency also impairs phagocyte function and cellular immunity. Folate has many roles in the human body including transfer of methyl groups to amino acids and DNA. Low folate intake is associated with increased risks of breast, lung (amongst former smokers), cervical, mouth and throat cancer. We aim to create transgenic maize plants with enhanced levels of the three key vitamins, A, E and folate, specifically targeting elucidation of fundamental mechanisms controlling expression of multiple transgenes co-introduced into an important crop plant. We have made plant transformation vectors utilizing endosperm-specific promoters for vitamins A and folate and the maize ubi1 constitutive promoter for vitamin E. We have cloned all genes required and we have used direct DNA transfer through particle bombardment to co-introduce all genes into maize. Here we describe our preliminary results and give a synopsis of the co-integration and expression of these genes into maize.

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Zn-responsive genes of *Thlaspi caerulescens*

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Zinc is an essential micronutrient required for a variety of functions in cellular metabolism, while being toxic at higher concentrations. Some plants have the ability to withstand, even accumulate, large quantities of Zn. The molecular mechanisms of plant heavy metal accumulation are not fully understood, however, it is an important trait as it determines both the micronutrient content and the toxic metal content of our food. *Thlaspi caerulescens* is a Zn and Cd hyperaccumulating species with several populations having differential metal tolerance, uptake and transport characteristics. We have previously isolated ca. 20 cDNA fragments which are differentially expressed in Zn exposure. Some of the fragments have a homology to *Arabidopsis thaliana* genes; the rest having only weak homology or representing unknown genes.

Among the Zn-responsive genes were two metallothioneins genes (*TcMT2* and *TcMT3*), with higher expression in Zn adapted population compared to population originating from uncontaminated soil. Moreover, yeast expressing TcMT2 or TcMT3 had a better capacity to grow at elevated Cu or Cd supply, implying that TcMTs are able to bind metals. However, little is known about the role of MTs in regard to Zn detoxification or buffering in plants. In this study, the expression of *TcMT*s in two intraspecies *T. caerulescens* crosses, segregating for Zn accumulation, was analyzed for possible co-segregation of MT expression and Zn accumulation capacity. The role of MTs in *T. caerulescens* will be further studied by using antibodies to localize the MT protein in the parental populations and in transgenic Arabidopsis plants expressing TcMT2 or TcMT3.

Biosynthesis of very long chain polyunsaturated fatty acids in chicory

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Very long chain polyunsaturated fatty acids (VLCPUFAs) are a declining resource, since they are obtained mainly from oily fish. The present investigation has evaluated the synthesis of VLCPUFAs in five cultivars, namely Brussels Witloof, Pan di Zucchero, Sponda da Taglio, Pain de Sucre and Poncho, of the leafy vegetable chicory (*Cichorium intybus*). Genes encoding enzymes of the ω3/6 Δ^8-desaturation biosynthetic pathways for the formation of C20 VLCPUFAs were inserted into leaf explants of chicory by *Agrobacterium*-mediated transformation of leaf explants. The genes for transformation encoded a Δ^9-specific elongating activity from *Isochrysis galbana* and a Δ^8-desaturase from *Euglena gracilis.* The genes were inserted into chicory either separately or in combination, and transgenic plants were selected on culture medium containing the herbicide glufosinate ammonium. PCR analysis showed the presence of the transgenes within the genome of selected plants, and the expression was confirmed by RT-PCR. Gas chromatography of fatty acid methyl esters extracted from freeze-dried leaves confirmed and quantified the production of the ω6 arachidonic acid precursors eicosadienoic acid (EDA, C20:2 $\Delta^{11,14}$) and dihomo-γ-linolenic acid (DGLA, C20:3 $\Delta^{8,11,14}$), and the ω3 eicosapentaenoic acid precursors eicosatrienoic acid (ETrA, C20:3 $\Delta^{11,14,17}$) and eicosatetraenoic acid (ETA C20:4 $\Delta^{8,11,14,17}$).

Production of eicosanoids in plants

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Eicosanoids, which include prostaglandins, thromboxanes and leukotrienes, are a large family of closely related lipid mediators. Prostaglandins (PGs) are classified into three series according to their 20-carbon polyunsaturated fatty acid precursors; series 1 PGs are derived from di-homo-γ-linolenic acid (DGLA, 20:3 ω-6), series 2 from arachidonic acid (AA, 20:4 ω-6) and series 3 from eicosapentaenoic acid (EPA, 20:5 ω-3). All series originate from the action of prostaglandin H synthase (PGHS) on the relevant fatty acids, and transgenic *Arabidopsis thaliana* plants producing DGLA, AA, and EPA have been constructed in our laboratory. We obtained cDNAs encoding the two isoforms of mouse PGHS, PGHS-1 and PGHS-2, and used PCR to subclone them in natural form, complete with signal peptide (PHGS-1SP and PGHS-2SP), or modified to lack the signal peptide (PGHS-1Ma). Their activities were then tested by expression in *Saccharomyces cerevisiae* using DGLA and AA as substrates, before proceeding to plant transformation. PGHS-2SP showed low activity in yeast relative to PGHS-1SP when AA was the substrate, but similar activity to PGHS-1SP when DGLA was the substrate. The removal of the signal peptide to produce PGHS1-Ma resulted in 50% decrease of the total activity of PGHS-1SP. *Agrobacterium*-mediated transformation was used to introduce all three PGHS genes into DGLA-producing *Arabidopsis* plants, and transformed plants were allowed to grow and segregate. Homozygous plant lines were analyzed for prostaglandin production by enzyme immunoassay. The results showed that PGHS-1-SP transformed plants produced the highest levels of PGs, with comparable levels present in the PGHS-2-SP transformants. Only extremely low levels of PGs could be detected in PGHS-1Ma transformed plants. Plant-produced PGs will now be purified to confirm their structures by NMR and mass spectroscopy.

Diversity analysis of nirvonic acid content, the ratio of mono to poly-unsaturated fatty acids, and DNA sequences in desaturase genes within the European collection of *Brassica oleracea*

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We have developed an approach to accessing the range of diversity within a large germplasm collection through the use of Diversity Fixed Foundation Sets (DFFS). *Brassica oleracea* has been subject to a range of selective pressures, breeding for different leaf, flower and root properties during the development of specialist crop types. Seed oil properties have not been subject to selection, unlike specialist oil crops such as *Brassica napus*. We have assessed the first 96 lines, from the *B. oleracea*, *B.napus* and where possible from the wild C genome DFFS collection (*http://www.brassica.info/diversity/diversity_sets.htm*), for variation in seed fatty acid composition. The *B. oleracea* lines, with some from the *B. napus* collection have also been screened for DNA sequence polymorphisms within the fatty acid desaturase genes *Fad1* and *Fad2*. These are known to be involved in the regulation of the degree of poly-unsaturation of the seed oil. The degree of variation uncovered in chemical and DNA-based assays, demonstrate the utility of the collection as a source of novel alleles for developing Brassica crops with oil profiles optimised for health benefits.

Expression levels of *OeCHPL* gene and tocopherol amount in different cultivars and feral form of olive (*Olea europaea* L.) plants

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Tocopherols are a group of lipid-soluble antioxidants, known as vitamin E, that both in plants and animals are required for many biological functions, mainly related to their antioxidant properties. These compounds are essential components of animal and human diet, being synthesized exclusively by photosynthetic organisms. Therefore, the genes involved in their biosynthesis may be useful tools as markers in breeding programmes or metabolic engineering to improve traits of agricultural crops and positively impact nutrition and human health.

Currently, the full set of genes involved in the tocopherol biosynthetic pathway are being identified in cyanobacteria and few higher plants and heterologously expressed. Since the major dietary sources of tocopherols are vegetable oils, the current study represents an initial step toward understanding the molecular bases of tocopherol biosynthesis in olive plants whose products, fruits and oil, are essential component of Mediterranean diet.

So far, a gene encoding the geranylgeranyl hydrogenase enzyme (CHLP) has been characterized in *Olea europaeae* cv. Carolea. This enzyme reduces free geranylgeranyl diphosphate to phytil diphosphate, providing the side chain to both tocopherols, plastoquinones and chlorophylls. The opening reading frame of 1395 bp encodes a deduced protein (*Oe*CHLP) of 464 amino acids that was 51,2 kDa and exhibited the maximal identity with *Nicotiana tabacum*. In order to relate gene activity to tocopherol synthesis, expression levels of *OeCHLP* have been evaluated by Q-PCR in the fruits of five olive cultivars with different tocopherol contents. One feral form was also investigated due to its wide utilisation as root-stock. In spite the growing knowledge about the individual enzymes required for tocopherol biosynthesis, the mechanisms that regulate the overall pathway and result in differential tocopherol content and composition during plant development remain poorly understood. For this reason, the level of *OeCHLP* expression has been monitored at different periods during fruit maturation. We demonstrated that the level of gene expression differed in relation to both cultivar and fruit developmental stage.

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Expression analysis of the lignin biosynthetic genes in Norway spruce using real-time RT-PCR

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Many of the enzymes involved in lignin biosynthesis are encoded by several genes, some of which can be related also to the biosynthesis of other phenylpropanoids. In this study, we explored gene expression across the whole lignin biosynthetic pathway in Norway spruce (*Picea abies* (L.) Karst.) using EST sequencing and quantitative real-time RT-PCR. Altogether 7189 ESTs were sequenced from a lignin forming tissue culture and developing wood of spruce, and clustered into 3831 unigenes. According to a tentative annotation, 2.4% of the unigenes were involved in monolignol biosynthesis. For most catalytic steps, several paralogous genes were found. Expression of the unigenes was studied in detail using real-time RT-PCR. Results highlighted the set of unigenes most likely responsible for monolignol biosynthesis in Norway spruce, also demonstrating that the same genes are expressed in all lignin-forming tissues. In contrast, peroxidases and laccases, together or separately responsible for lignin polymerisation, had distinct expression profiles in different tissues. Induction of lignin biosynthetic gene expression in compression wood and after infection with *Heterobasidion annosum* was also studied. Compression and vertically grown wood were similar, as only a few genes were statistically significantly induced in compression wood. Pathogen infection resulted in a general up-regulation of the monolignol biosynthetic pathway, and a specific induction of a few peroxidase genes. *PaPAL2*, *PaPX2* and *PaPX3* were induced in both compression wood and pathogen infection, suggesting a general stress-induced function.

Unravelling the full complement of phytase encoding genes from wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.)

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Phytic acid (phytate, $InsP_6$, *myo*-inositolphosphate 1,2,3,4,5,6-hexakisphosphate) is known as the primary storage form of phosphate and inositol in plant seeds. Moreover, phytic acid is considered to be the single most important anti-nutritional factor for the bioavailability of minerals in human nutrition. During germination, phosphate and minerals are released from the phytic acid due to the activity of the hydrolytic enzymes 3- and 6-phytase (*myo*-inositol hexaphosphate 3- and 6-phosphohydrolase). However, in most dry seeds and in the digestive tract of humans, there are insufficient or no phytase activities. For human nutrition the low digestibility of phytate has severe consequences. Phytate efficiently chelates a range of important minerals such as iron and zinc, and inhibits their bioavailability. This is in particular a problem in developing countries where the nutrition of the poor population almost exclusively is based on cereals.

In spite of their importance surprisingly little is known about plant phytases. In our current work, we are unravelling the full complement of barley and wheat phytase genes and enzymes. We report on the cloning and characterization of cDNAs encoding one of the groups of enzymes with phytase activity, the multiple inositol phosphate phosphatase (MINPP). Four wheat cDNA's (*TaPhyIIa1*, *TaPhyIIa2*, *TaPhyIIb* and *TaPhyIIc*) and three barley cDNA's (*HvPhyIIa1*, *HvPhyIIa2* and *HvPhyIIb*) were isolated. The ORF´s were from 1548–1554 bps and the level of homologies between the barley and wheat proteins ranged from 90.5–91.9%. All cDNAs contained an N-terminal signal peptide encoding sequence while a KDEL-like sequence, KTEL, was present at the C-terminal indicating that the enzyme is targeted to and retained within the endoplasmic reticulum. Expression of *TaPhyIIa2* and *HvPhyIIb* in *Escherichia coli* revealed that the MINPP's posses a significant phytase activity with narrow substrate specificity for phytate. The pH and temperature optima for both enzymes were 4.5 and 65 ºC and the Km's for phytate 246 and 334 µM for the wheat and barley recombinant enzymes respectively. The enzymes are inhibited by several metal ions, in particular copper and zinc. The cDNA's showed significantly different temporal and tissue specific expression patterns during seed development and germination. With the exception of *TaPhyIIb*, the cDNA's were present during late seed development and germination. Moreover we recently isolated three and two isogenes of purple acid phosphatases (PAP's) from wheat and barley respectively. Expression in *E. coli* and *Pichia pastoris* revealed that the corresponding proteins are significant phytases with Km's for phytate on 182 µM, temperature optimum ~40 ºC and dual pH optima on 4,5 and ~7. The specific activity of the PAP phytases with phytic acid as substrate is 20 µmol/min×mg, around 20 times higher than the MINPP phytases.

Improving oat β-glucan content by biotechnological methods

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Oat (*Avena sativa L.*) products lower the blood cholesterol level, a risk factor in heart diseases. Moreover, β-glucan also helps to normalize the postprandial blood glucose level and reduces the risk of colon cancer. These health benefits are mainly associated with high levels of mixed-linked (1,3)-(1,4)-β-D-glucan, a dietary fiber of which the main part is soluble. The amount of β-glucan is dependent on the genotype and thus can be increased by cultivar breeding. In this study, modern biotechnology tools were applied in order to breed high β-glucan oat cultivars for food and processing purposes.

To understand the inheritance of β-glucan content in oat and to enable DNA-marker-assisted selection in oat cultivar breeding, a genetic linkage map was constructed for a Nordic oat cross. An Aslak × Matilda mapping population of 137 totally homozygous DH-lines was produced by anther culture. The linkage map consisted of over 600 PCR-based DNA-markers, including microsatellites, RAPDs, REMAPs, ISSRs, SRAPs and AFLPs. The QTL analysis showed two QTLs associated with β-glucan content. Together they explained about 37% of the variation in the DH lines. In both chromosomal regions alleles from Aslak had a favorable effect on β-glucan content. On the basis of the results, markers tightly linked to the genes themselves may be developed for DNA-marker assisted selection of high β-glucan content containing oat breeding lines. QTLs were located also for other traits of interest. Another aim was to modify the β-glucan content of Finnish oat cultivars through genetic engineering. Embryogenic cell cultures were started from mature embryos of oat cultivars Aslak, Veli, and Kolbu. A microbial 1,3-β-glucan synthase was transferred to oat cell lines by particle bombardment and transgenic plants were regenerated. The expression of the microbial gene in transgenic cell cultures was demonstrated by semi-quantitative RT-PCR. The 1,3-1,4-beta-glucan amounts were reduced and the molecular weight of the mixed-linked β-glucan differed in some transgenic seed lines when compared to non-transgenic control seeds. The analyses of 1,3-beta-glucan contents of transgenic cell lines and seeds is ongoing. A basis exists for modifying oat β-glucan contents through genetic engineering.

Xanthophyll content modifications in leaf and fruit of transgenic tomato plants overexpressing the beta-carotene hydroxylase (*CrtR-b2*) gene from *S. lycopersicum* (cv. Red Setter)

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Transgenic tomato lines, over-expressing the tomato beta-carotene hydroxylase cDNA (*CrtR-b2*) driven by *CaMV 35S* promoter, were assayed for ability to accumulate beta-carotene-derived xanthophylls. Two out of eight transformants analyzed by quantitative Real-Time PCR showed a huge increase in the CrtR-b2 transcript in leaves, petals and fruits. The T1 progenies of these two clones manifested an altered phenotype when grew in high light conditions. Specifically leaves presented a diffuse mosaic consisting of green and white areas. Analogous anomalies were observed on the stem, branches, petioloes and pedicels. Plants bloomed regularly, the flowers presented the usual yellow colour and had a high percentage of fruit set. The fruits firstly appeared lightly yellow/green instead of brilliant green and at the ripening stage acquired a red/orange colour. The leaf carotenoid HPLC analysis of transgenic plants showed an increase of the neoxanthin, anteraxanthin and violaxanthin contents in comparison to the control. The same situation was observed in young fruits where the carotenoid analytical determination revealed the presence of the neoxanthin absent in the wild type. Neoxanthin level in transgenic fruits decreased progressively during the ripening process disappearing at mature stage. This reduction is probably related to the plastid differentiation involving the change of the chloroplasts present in young fruit to the chromoplasts typical of the ripe fruit. Further investigations are in progress to better understand this behaviour.

Comparison of transgenic *Gerbera hybrida* lines and traditional varieties shows no differences in cytotoxicity or metabolic fingerprints

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Genetic modification using gene transfer is still controversial at least in Europe. One major concern is how genetic modification of single genes affects other genes and thus the metabolism of the plant. In this study, 227 genetically modified lines of the ornamental plant *Gerbera hybrida* and 42 non-GM gerbera varieties were used to investigate changes in secondary metabolism. The cytotoxicity of GM and non-GM gerbera extracts was evaluated on human cell lines derived from lung, liver, and intestinal tissues using the cell proliferation reagent WST-1. In addition, metabolic fingerprints of gerbera extracts were identified using thin-layer chromatography and analysed statistically. The results from cell viability assays indicate that the safety profile for GM gerbera lines is similar to the viability pattern for regular varieties. None of the extracts were toxic. 33 different quantified area values were gained with TLC. No new compounds unique to GM lines were observed. Quantified area values were normalized and analysed by principal component analysis (PCA), the nearest neighbour classifier, and Fligner-Killeen test. With PCA, no separation between GM gerbera lines and varieties could be demonstrated. In the nearest neighbour classifier, 53.6% of the samples found the expected neighbour based on the gene constructs used for transformation or the observed phenotype. With Fligner-Killeen test, we studied if the amounts of compounds vary more in GM gerberas than in varieties. We expected that there is more variation among varieties than in GM lines that have been made from one variety. In the most cases, there were no statistically significant differences between the varieties and GM lines or there were more variation among the varieties than in the GM lines. The variance of one compound was statistically significantly bigger in transgenic gerbera lines than in varieties.

RECOMBINANT PROTEINS
E1–E8

Recombinant Amb a 1 expression

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The number of identified pollen allergens, especially homologues belonging to the group of pectate lyases, is constantly rising. The main allergenic protein of common ragweed (*Ambrosia artemisiifolia*) Amb a 1 resembles the most prominent specimen. The major allergens from pollen of cedar, juniper and cypress species Jun a 1 (*Juniperus ashei*), Cry j 1 (*Cryptomeria japonica*), Cup a 1 (*Cupressus arizonica*) and Cha o 1 (*Chamaecyparis obtuse*) as well as Art v 6 from Mugwort (*Artemisia vulgaris*) also belong to the family of pectate lyases. Pectate lyases themselves resemble a large group of enzymes not only expressed in ripening fruits and in plant pathogenic bacteria, but also in pollen. These enzymes are able to digest the pectin or pectate envelope of plant cells. Although enzymatic activity is not shown for all above mentioned allergens, the high homology on aminoacid level and the cross-reactivity of patient´s sera especially within the conifers unify this group.

In the U.S. about 50% of all allergic individuals (including patients with food, dust, mite and other allergies) suffer from ragweed pollinosis (hay fever). This shows the magnitude of the ragweed problem. Since ragweed already started invading parts of France, Hungary, Germany, Finland and Austria, this very potent, allergy provoking plant will soon cause big problems in Europe, too. Accurate standardization of natural pollen extracts is a difficult task. Thus there is a need for reliable and effective diagnostic and therapeutic tools for allergy treatment. Therefore the production of soluble recombinant allergens of the pectate lyase group is an important task. For this purpose we were evaluating the plant-based expression systems for Amb a1.

Generation of *Nicotiana benthamiana* lines for the production of recombinant proteins lacking plant specific N-glycan epitopes

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Plants, and in particular *Nicotiana ssp*., are considered as a promising alternative production system for pharmaceutically relevant proteins. However, the inability of plants to perform authentic mammalian *N*-glycosylation may cause a limitation in this respect. A major concern is the presence of the β1, 2-xylose and core α1, 3-fucose residues on complex *N*-linked glycans as these structures are immunogenic in mammals. In our attempts towards humanisation of plant *N*-glycans we have generated *N. benthamiana* mutants that show RNAi targeted expression of β1, 2-xylosyltransferase (XylT) and core α1, 3-fucosyltransferase (FucT), the two enzymes responsible for the attachment of these glycan epitopes. This process involved the identification of XylT and FucT cDNAs and the subsequent transformation of *Nicotiana benthamiana* with RNAi constructs thereof. Transformed lines exhibited complex *N*-glycans, carrying minimal amounts of xylose and core α1, 3-fucose residues, respectively, as analysed by immunoblotting and MALDI-TOF-MS. RT-PCR experiments showed a significant reduction of XylT and FucT expression in transformed plants. Reduction of xylosylation and fucosylation remains stable for at least three generations. RNAi lines are viable and revealed no obvious morphological phenotype under standard growth condition and during the transient expression process.

A monoclonal antibody was transiently expressed in wild type (wt) plants and RNAi lines. Whereas purified IgG derived from wt lines displayed complex *N*-glycans fully decorated with xylose and core α1, 3-fucose, RNAi derived IgG carries complex *N*-glycans with minor amounts of xylose or fucose epitopes.

Engineering of the *N*-glycosylation pathway in the model plant *Arabidopsis thaliana*

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The presence of non-human *N*-glycan epitopes as well as the absence of sialic acid residues may cause limitations for plants to be used as factories for therapeutically relevant glycoproteins. Therefore, approaches have to be developed to eliminate non-human *N*-glycan structures and to reconstruct the mammalian sialic acid pathway in host plants. In order to eliminate the plant specific attachment of β1,2-xylose and core α1,3-fucose residues to *N*-glycans, *Arabidopsis thaliana* plants with disruptions in the respective glycosyltransferase genes were generated. Endogenous glycoproteins from these triple knockout plants are devoid of immunogenic *N*-glycans. Importantly, the knockout plants synthesize predominantly *N*-glycans with terminal β-*N*-acetylglucosamine residues, which are a prerequisite for the further restoration of human-type *N*-glycosylation. A monoclonal antibody expressed in these plants carries such mammalian-like *N*-glycans without β1,2-xylose and core α1,3-fucose and is indistinguishable from its mammalian counterpart.

As a first step towards *N*-glycan sialylation in *A. thaliana*, we have independently transformed plants with the six missing genes required for sialylation of nascent glycoproteins: UDP-GlcNAc epimerase/ManNAc kinase (GNE), Neu5Ac-9 phosphate synthase (SAS), CMP-Neu5Ac synthetase, the CMP-Neu5Ac transporter (CST), β1,4-galactosyltransferase (GalT) and α(2,6)-sialyltransferase (ST). All six genes were successfully expressed *in planta*. Furthermore, *in vitro* assays confirmed that recombinant GNE, SAS, CST, GalT and ST are functionally active when produced in plants. This proves the feasibility to express all mammalian proteins required for *N*-glycan sialylation *in planta,* which is a first step towards assembly of a functional sialylation pathway in plants. Thus, our results demonstrate that humanization of plant *N*-glycosylation is possible which improves the prospect of exploiting plants as factories for the production of recombinant glycoproteins.

Production of recombinant human hepcidin in carrot (*Daucus carota*) hairy roots

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Hepcidin is a 25-amino acid disulphide-rich peptide secreted by the liver. Hepcidin has been shown to control body iron homeostasis by suppressing intestinal iron absorption and reticuloendothelial iron recycling. Genetic iron overload (hemochromatosis) is characterized by excessive iron absorption from the diet followed by toxic iron deposition in vital organs. Abnormally low levels of hepcidin are involved in the development of the disease including types with non mutated hepcidin gene (*Hamp*). The use of recombinant hepcidin in the treatment of hemochromatose could thus become the starting point of a new therapeutic approach.

The goal of our work is to explore the possibility to produce human recombinant hepcidin by plant hairy roots. Hairy roots emerge from plant tissues infected by the phytopathogen *Agrobacterium rhizogenes*. They are fast-growing tissues which do not need phytohormones and can be cultured in bioreactors using simple (thus cheap) media. The expression cassette designed to overexpressed hepcidin in carrot (*Daucus carota*) hairy roots includes a duplicated 35S promoter followed by the coding sequence of a secretion signal peptide from *Arabidopsis* fused in frame to the coding sequence of hepcidin. Eventually, the cassette includes the His tag sequence to produce the N-terminally tagged hepcidin. Carrot discs were transformed by *Agrobacterium rhizogenes* harboring the expression cassette in a binary vector, and transgenic hairy roots were selected on kanamycin-containing medium. His-tagged hepcidin was purified by immobilized Ni^{2+} affinity chromatography from the culture medium or from a crude protein extract of hairy roots, and analyzed by tricin SDS-PAGE electrophoresis. The antimicrobial activity of recombinant hepcidin *in vitro* would allow monitoring the production of a biologically active peptide before to test its *in vivo* activity on model animals.

Improving heterologous protein expression in *Nicotiana tabacum* culture cells

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We have compared expression of a monoclonal antibody, LO-BM2, in *Nicotiana tabacum* plants and suspension cells (BY2). This chimeric rat/human antibody (IgG1) has the potential to prevent hyperacute xenograft rejection. Both chains were expressed, correctly folded, assembled and secreted in the extracellular medium. In both expression systems, the construct bearing the heavy and the light chain genes in tandem orientation led to higher expression level than the construct in inverted orientation. LO-BM2 content reached 0.2% of total soluble proteins in both systems. We also showed that purified LO-BM2 was able to correctly bind primate anti-pig IgM at the surface of pig lymphocytes.

However, in both expression systems, the presence of smaller fragments of the heavy chain indicated that this chain is particularly sensitive to degradation. We therefore decided to identify the extracellular proteases responsible for LO-BM2 cleavage with the aim of preventing their expression by RNA silencing. Although more abundant and diversified, leaf intercellular fluid (IF) proteases appeared less active on antibodies than their culture medium counterparts. Mass spectrometry analysis combined with *in vitro* assays allowed us to identify three proteases belonging to the subtilisine-like serine protease family (S8 class) in both leaf intercellular fluid and culture medium. Close homologs in tomato and *Arabidopsis* presumably participate in the plant defence. In addition, a cysteine peptidase of the papain-like family (C1 class), NtCP-23, was only identified in the leaf intercellular fluid. cDNA clones were obtained from these proteases and specific sequences are currently being used to prevent their expression in transgenic plants by RNA intereference.

Production of viral (HIV-1/Pr55^{gag} and HPV-16/L1) antigens in transplastomic tobacco plants

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Plants are a promising production platform of therapeutic proteins. Both HIV-1/Pr55^{gag} and HPV-16/L1 antigens, however, are difficult to produce in plant cells. Herein, we report their expression in transplastomic plants. The transformation of the plastid genome generally shows high protein expression levels, precise transgene insertion by homologous recombination, no gene silencing and transgene containment.

Gag, the major structural protein of HIV-1, is capable of assembling in Virus-Like Particles (VLPs), eliciting both humoral and cellular immune response, and is therefore a primary candidate for the development of an effective and safe vaccine. In transplastomic plants, western analyses with antibodies against p24 and p17 Gag subunits detected the presence of a strong band of about 40 kDa consisting of the N-terminal matrix (p17) and the central capsid (p24) domains. Hence, plastidial proteases process the Pr55^{gag} polyprotein similarly to the viral protease in human cells. In our laboratory, *N. benthamiana* plants, agroinfiltrated with either the full *gag* gene or its subunits, showed similar results (Ferraiolo et al., in preparation). The plastid-expressed Gag protein was also able to assemble into particles resembling VLPs produced in baculovirus and *E. coli* systems.

L1 gene codes for the major HPV capsid protein, which also forms VLPs. Several vectors, containing either the wild-type L1 sequence or a sequence changed according to the plastid codon usage, were constructed. L1 expression was regulated by strong plastid promoters and different 5'-UTRs. Recombinant L1 protein was detected by western blots only when translated plastid sequences were added upstream of the viral gene (Downstream Box – DB). Results of capture ELISA assays, carried out with MAbs recognizing conformational epitopes, suggest that the L1 protein produced in transgenic plastids is able to assemble into VLPs.

Transgenic tomato as a bioreactor for production of human fibroblast growth factor

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Plants as bioreactors of pharmaceutical proteins are safer comparatively to microorganisms, animals and animal tissues because the lack of human pathogens, prions, oncogenic DNA sequences and endotoxins. Tomatoes are widespread vegetable crop suitable for use as bioreactor in molecular farming area because the methods of genetic transformation via *Agrobacterium* are well developed. In the last twenty years tomatoes are object of investigation in the area of cell and tissue cultures and genetic manipulations. Human acidic fibroblast growth factor takes part in stimulation of DNA syntesis and in the proliferation of a wild variety of cell types including fibroblasts, epithelial and endothelial cells, smooth muscle cells, myoblasts etc. aFGF plays important role in varios stages of development and morphogenesis and also in angiogenesis and wound healing processes. This data present the wide variety of application of aFGF in injured blood vessels recovery, ischemia and ulcer healing, slowly recovering skin wounds.

As a result of the successfully applied method for *Agrobacterium*-mediated transformation, tomato plants carrying the gene for human fibroblast growth factor acidic in their genome have been developed. Several transgenic lines from wild tomato species *Lycopersicon pennellii* as well as *L. esculentum* cv. Bela plants have been obtained. *Agrobacterium tumefaciens* LBA4404 strain supplemented with a constitutive *vir*G mutant gene on a compatible plasmid for very efficient T-DNA transfer to plants was used. The binary vector – kindly provided from prof. Wang from Institute of Genetics and Cytology in The Northeast Normal University, China – contains selective marker gene for phosphomannose isomerase which determines resistance to the monosaccharide mannose which is less widespread in plant kingdom; and the gene for human acidic fibroblast growth factor. The obtained transgenic tomato plants and T1 progeny were analyzed with PCR and indirect ELISA tests with antibodies for haFGF. Further molecular analyses as well as allergenicity and toxicity tests are in progress. The initiation of cell suspension cultures from transgenic tomato plants is discussed.

Production of recombinant gelatin in transgenic barley grain

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The large-scale production of recombinant DNA-based mammalian proteins in plants can provide a safe, animal-component free, homogenous and cost-effective source for those proteins that are currently derived from animal or human sources. The aim of our project is to develop a production system for an industrial protein, gelatin, utilizing barley grain. Gelatin is an important component in many products in the food, photographic, and pharmaceutical industries. In this study a 50,000 Dalton fragment of the human collagen I alpha 1 chain was accumulated in transgenic barley grain. The monocot expression optimized cDNA coding for this recombinant gelatin was fused to a signal sequence and the ER retention signal HDEL. Gene expression was controlled under (1) a constitutive maize ubiquitin promoter, (2) a seed-specific rice glutelin promoter or (3) a germination dependent barley α-amylase promoter. The gene constructs were co-transformed with the *bar* or *hyg* selection marker genes into immature barley embryos either by particle bombardment or by *Agrobacterium*-mediated delivery. The accumulation levels of the recombinant gelatin in the barley grains varied depending on the promoter used.

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Plants for Human Health in the Post-Genome Era
PSE Congress

Abstract

For the international congress 'Plants for Human Health in the Post-Genome Era' organized on behalf of the Phytochemical Society of Europe (PSE) and VTT Technical Research Centre of Finland almost 200 distinguished scientists from academia and industry are gathering in Helsinki from the 26th until the 29th of August 2007. This is the first PSE regular congress after the 50 years celebration of the Society.

The scientific programme covers all aspects of modern plant science in relation to human health in eight consecutive sessions: Opportunities for plant biotechnology, health benefits from plants, emerging technologies in plant research, plants as factories for pharmaceuticals (one session each for secondary metabolites and proteins), large-scale production for industrial applications and future outlook. Central aim is to stimulate interdisciplinary discussion. Thirty outstanding speakers from various fields are presenting and discussing their latest results. Further contributions are made by short oral presentations selected from the submitted abstracts and by poster presentations. The registered participants come from 33 countries.

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