cost
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PlantEngine
COST Action FA1006

Book of Abstracts

Final Conference

Challenges and prospects in PNP metabolic engineering and production

Sorrento, Italy
April 15th-17th, 2015

UNIVERSITÀ DEGLI STUDI DI SALERNO
DIFARMA
Dipartimento di Farmacia




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PlantEngine COST Action FA1008	**Challenges and prospects in PNP metabolic engineering and production**
Date	April 15th-17th, 2015
Site	Hotel Parco dei Principi, Sorrento (Italy)

Wednesday, April 15th 2015		
10:00-13:00	**Registration and poster setup**	
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10:05-10:25	**Mariana Sottomayor, PO** *A molecular toolbox to engineer the metabolic flux of the anticancer alkaloids from Catharanthus roseus*
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11:20-11:40	**Jacob Pollier, BE** *Plant roots as a perfume? Overexpression of a MYB transcription factor leads to methyl anthranilate emission in M. truncatula hairy roots*
11:40-12:00	**Antonella Leone, IT** *Driving metabolic flux towards high content of abietane-type diterpenes in Salvia sclarea hairy roots*
12:00-12:20	**Turgay Unver, TR** *Transcriptomics and metabolomics approaches towards better understanding of BIA biosynthesis in opium poppy*
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17:25-17:45	**Lorena Almagro, ES** *Diadenosine triphosphate is a novel factor which synergistically with cyclodextrins enhances the biosynthesis of trans-resveratrol in suspension cultured cells of Vitis vinifera cv Monastrell*

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10:20-10:40	**Giovanni Giuliano, IT** *Saffronomics: Transcriptomics-based dissection of the saffron stigma apocarotenoid pathway*
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11:30-12:00	Key-note lecture **Julian Ma**, St. George's University of London, London, **UK** *Plant made recombinant pharmaceuticals - where is the field?*
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Session I

Capturing secrets from nature

Key-note lecture

Carotenoid biosynthesis BC and AC, and genetic pathway engineering for optimizing carotenoid production

Gerhard Sandmann
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The carotenoid pathway was one of the first targets for genetic manipulation towards higher productivity or synthesis of novel compounds. For successful and productive pathway engineering, it is essential to know as much as possible about the biochemical background, about individual pathway reactions, organization, genes and enzymes involved and regulation. In addition, all necessary tolls including useful genes and efficient transformation protocols have to be acquired. Knowing the physiology of the metabolites from a given biosynthesis pathway may help to fortify plants against harsh environmental conditions. After giving an overview on nutritionally and economically important carotenoids and the development of the molecular biology of carotenogenesis, several efforts will be presented exemplifying of how in microorganism and crop plant carotenoid biosynthesis was enhanced or extended to novel structures. The cloning strategies will be discussed and also the challenge and problem to compete economically with chemical synthesis, although the use of renewable resources is highly desired.

O-1

Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations

Nogueira M[1], Mora L[1], Enfissi E[1], Bramley P M[1], and Fraser P D[1]
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Metabolic engineering of the carotenoid pathway in recent years has successfully enhanced the carotenoid contents of crop plants. It is now clear that only increasing biosynthesis is restrictive, as mechanisms to sequestrate these increased levels in the cell or organelle should be exploited. In this study, biosynthetic pathway genes were overexpressed in tomato (Solanum lycopersicum) lines and the effects on carotenoid formation and sequestration revealed. The bacterial Crt carotenogenic genes, independently or in combination, and their zygosity affect the production of carotenoids. Transcription of the pathway genes was perturbed, whereby the tissue specificity of transcripts was altered. Changes in the steady state levels of metabolites in unrelated sectors of metabolism were found. Of particular interest was a concurrent increase of the plastid-localized lipid monogalactodiacylglycerol with carotenoids along with membranous subcellular structures. The carotenoids, proteins, and lipids in the subchromoplast fractions of the transgenic tomato fruit with increased carotenoid content suggest that cellular structures can adapt to facilitate the sequestration of the newly formed products. Moreover, phytoene, the precursor of the pathway, was identified in the plastoglobule, whereas the biosynthetic enzymes were in the membranes. The implications of these findings with respect to novel pathway regulation mechanisms are discussed.

O-2

Development of an automated gene discovery framework to highlight pivotal plant metabolic and genomic features

Tzfadia O[1,2], Van de Peer[1,2,3]
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A biological pathway is the set of molecular entities (genes) involved in a given biological process and the interrelations among them. Although current knowledge about some biological pathways is substantial and useful for systems-level analyses, not all genes that participate in and affect function of these pathways are known. Prime examples of information gaps are biosynthetic pathways leading to secondary metabolites in plants. Filling the gaps in our current knowledge about biological pathways and gene discovery is a fundamental challenge. The rapidly increasing availability of high-throughput experimental 'omics' data sets (e.g., genomics, transcriptomics, proteomics, metabolomics etc.) serves as a platform for maximizing computer and mathematical techniques for enhancing the identification of key player genes in metabolic and biological pathways.

For this 'data-driven philosophy' to work however, several key challenges need to be addressed. First, structural and functional annotation as well as pathway analysis needs to be performed in a reliable, automated manner, without much further need for manual intervention by expert users. As annotated functional elements and pathways form the biological 'features' of an organism, their accurate identification is imperative. Second, these features (and combinations thereof) need to be analyzed and compared to those of other organisms to uncover biologically relevant characteristics. Finally, the developed algorithms and implementations should be able to deal with large amounts of data, for which analysis can typically not be

performed on a single laptop or workstation. The use of parallel and distributed computing techniques is therefore essential.

Here, we are developing an automated cost-effective-high-throughput-gene-discovery framework that, based on data mining and smart integration of different sources of information, can fill gaps in metabolic pathways and transferring information from model (Arabidopsis, tomato, rice, maize, etc.), to non-model plant species.

O-3

Biochemical Characterization of novel CCDs from Saffron

Sarah Frusciante[1], Gianfranco Diretto[1], Paola Ferrante[1], Marco Pietrella[1], Salim Al-Babili[2], Lourdes Gomez-Gomez[3], and Giovanni Giuliano[1]

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Saffron stigmas are used as a spice since the Minoan civilization, due to their content of different valuable metabolites that include three apocarotenoids (crocetin, picrocrocin and safranal) that contribute to the red color, bitter taste and aroma of saffron and whose synthesis occurs during early stigma development. This pathway is initiated by a carotenoid cleavage reaction catalyzed by a CCD (Carotenoid Cleavage Dioxygenase). Conflicting data have been reported about the identity of this enzyme and its substrate (β-carotene vs zeaxanthin). Using RNA-Seq profiling, of 6 different saffron stigma developmental stages, from immature to fully developed, we identified 7 different CCDs, CCD1, CCD4a, CCD4b, CCD4c, CCD7, CCD2 and ZCD, the previously described as the biosynthetic candidate enzyme. All the identified CCDs were expressed in *E. coli* cells engineered to accumulate different carotenoids (lycopene, β-carotene and zeaxanthin) and tested both *in bacterio* and *in vitro*. We confirmed the symmetric cleavage activity for CCD1 at 9,10 (9',10') acting on β-carotene, lutein and zeaxanthin and the asymmetric cleavage at 9,10 position acting on apocarotenoids and hydroxy-apocarotenoids. We could not detect any cleavage activity for CCD4a, CCD4c and CCD7, while CCD4b seemed to cleave β-carotene in a different position from the 9,10 (9',10') reported previously. Finally, CCD2, mostly expressed during the early stages of the stigma maturation, cleaved zeaxanthin, but not lycopene or β-carotene at the 7,8 and 7'8' positions, yielding crocetin dialdehyde. This activity was confirmed through transient expression in maize kernels, where the synthesis proceeds to crocetin, likely due to an endogenous aldehyde dehydrogenase activity. In vitro reactions performed with different substrates gave a clear idea of the

stereospecificity of the enzyme. In contrast to a previous report, ZCD did not show any cleavage activity, consistent with it being an N-truncated CCD, lacking one blade of the β-propeller structure conserved in all CCDs. These results suggest that CCD2 is the enzyme responsible for the cleavage step leading saffron apocarotenoid biosynthesis.

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O-4

Iridoid Synthase Activity Is Common among the Plant Progesterone 5β-Reductase Family

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Catharanthus roseus, the Madagascar periwinkle, synthesizes bioactive monoterpenoid indole alkaloids, including the anti-cancer drugs vinblastine and vincristine. The monoterpenoid branch of the alkaloid pathway leads to the secoiridoid secologanin and involves the enzyme iridoid synthase (IS), a member of the progesterone 5β-reductase (P5βR) family. IS reduces 8-oxogeranial to iridodial. We show that IS belongs to a family of six *C. roseus* P5βR genes. Characterization of recombinant CrP5βR proteins demonstrates that all but CrP5βR3 can reduce progesterone enantioselectively to 5β-pregnane-3,20-dione and can thus be classified as P5βRs. P5βRs are described to be involved in cardenolide biosynthesis in cardenolide containing plants, such as *Digitalis lanata* and *Erysimum crepidifolium.* Three of the P5βR isolated from *C. roseus*, namely CrP5βR1, CrP5βR2, and CrP5βR4, can also reduce 8-oxogeranial, pointing at a possible redundancy with IS (corresponding to CrP5βR5) in secoiridoid synthesis. We cloned a set of P5βR genes from angiosperm plant species not known to produce iridoids and demonstrate that the corresponding recombinant proteins are also capable of using 8-oxogeranial as a substrate. This suggests that IS activity is intrinsic to angiosperm P5βR proteins and has evolved early during evolution.

The wide substrate range that includes also small toxic molecules can be interpreted as the evolution of different physiological functions for different P5βRs *in planta*. Besides the role in iridoid and cardenolide biosynthesis, a role in the detoxification of small toxic molecules, such as methylvinylketone, is also possible.

Key-note lecture

Making new molecules

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Plants produce a tremendous array of natural products, including medicines, flavours, fragrances, pigments and insecticides. The vast majority of this metabolic diversity is as yet untapped, despite its huge potential value for humankind. The recent discovery that genes for the synthesis of different kinds of natural products are organised in clusters in plant genomes is now opening up opportunities for systematic mining for new pathways and chemistries. Improved understanding of the genomic organization of different types of specialized metabolic pathways will shed light on the mechanisms underpinning pathway and genome evolution. It will also provide grist for the synthetic biology mill.

Session I Posters

Capturing secrets from nature

P-1

Phenolic composition of cloudberry and arctic bramble cell suspension cultures

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Chemical composition of Nordic wild berries is unique because of the challenging, short growing season with abundant light and often high variations between night and day temperatures. Thus in the Nordic latitude significance of secondary compounds, such as phenolics in plants is emphasised, as these compounds are primary produced for plant defence in environmental stress conditions. Berries in the genus *Rubus*, especially cloudberry (*R. chamaemorus)* and arctic bramble (*R. arcticus*) are unique Nordic berries, and important raw material for food, beverage and cosmetic industry. However, these berry species are very sensitive towards changing climate. Also effective modern soil and forest cultivation techniques are harmful and restricting their growth. Thus biotechnology might offer respectable approach to advantage these species in the future in sustainable and eco-friendly way.

We have established cell cultures of cloudberry and arctic bramble from ex-plants originated from the pure Nordic nature. Scale-up and down-stream processes were also developed for these cell cultures for controlled production and harvesting of material of interest. Whole biomass or extracts prepared from that can be used for various industrial applications, such as cosmetics. We have recently studied phenolic composition of these cell cultures. Interestingly, their biosynthetic capacity seems to be different from that of the berry fruits, indicating varied bioactivities exploitable in industrial applications related to health and wellbeing. Phenolic composition of the cell cultures will be discussed in detail.

P-2

Tissue culture and physical elicitation for production of secondary metabolites in *Salvia* species

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Salvia, is the largest genus of Lamiaceae, comprising aromatic, officinal and medicinal species. Extracts secreted from glandular trichomes of plants belonging to this family are a valuable source of biologically active secondary metabolites as non volatile and volatile compounds (VOCs). Different efforts have been done to increase the content of sage-derived bio-active compounds for pharmaceutical purposes leading to a high pressure on wild over-utilized populations. The use of plant tissues and cell cultures may represent an alternative strategy for production of active compounds without destroying indigenous biodiversity. This study deals with the establishment of *in vitro* manipulation protocols, elicitation experiments and further determination of secondary metabolites in two selected *Salvia* species having a diverse origin and phytochemical profile. *Salvia dolomitica* Codd, a particularly fragrant sage native to South Africa, was selected due to its aroma caused by VOCs emission. In our work, the chemical composition of its essential oils was characterized in differently grown plants since *S. dolomitica* can be easily and quickly propagated *in vitro*. Our results showed that in vitro technology modulates the accumulation of specific secondary metabolites like myricene, limonene and α-pinene. Moreover, by applying a physical elicitor on in vitro cultured plantlets as light intensity, which may affect trichomes number and morphology, we demonstrated that elicitated plants have a quanti-qualitative diverse phytochemical profile. Interestingly, the yield of essential oils was higher in high light treated plants compared to the ones cultivated *in vivo* and *in vitro*. Secondly, we focus our attention on a South America derived sage, *Salvia corrugata*. Extracts from trichomes secreted exudates leaded to the isolation of two diterpenes, demethyl fructiculin (SCO-1) and fructiculin (SCO-2) whose

phytochemical activities have been previously demonstrated. In our study we were interesting in address whether high light may affect the accumulation of this compounds in *in vitro* grown *S. corrugata* plants. Surprisingly, the applied elicitor negatively affect the accumulation of SCO-1 and -2 maybe affecting the expression level of diterpenoids biosynthetic genes or trichomes distribution. Taking all together our data strongly suggest that combining micropropagation and elicitation might be a valid strategy to modify the production of bioactive compounds derived by clonal plantlets and shed light on the possible use of *S. dolomitica* micropropagated plants as model to study the impact of climatic factors (e.g.temperature, photoperiod) on VOCs emission in aromatic plants.

P-3

Studying the β-1,3 glucanase gene from medicinal herb sundew *Drosera rotundifolia* L.

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The common sundew (*Drosera rotundifolia* L.) is a carnivorous medicinal plant the gene pool of which is poorly studied. We were interested in research for defense-related genes, primarily from the group of PR family (Pathogenesis-related proteins). The complete sequence of the gene encoding β-1,3 glucanase was isolated. Analyses *in silico* showed the encoded protein is a putative extracellular, acidic glucanhydrolase of ~37 kDa. It bears all typical features of glucanases described in other plant species. Gene promoter activity was studied in transgenic tobacco using the *gus*-gene reporter system. We assume the β-1,3- glucanase gene plays role in elongation growth and remobilization of nutrients. The gene is inducible under certain types of abiotic stress like dark and drought, but not mechanical wounding or cold. Transformation protocol for sundew was established to perform further functional studies of the gene.

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P-4

Metabolite profiling of *Dioscorea* (yam) species reveals underutilised biodiversity and foliage as a source of high-value compounds

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Yams (*Dioscorea spp.*) are a multispecies crop with production in over 50 countries generating ~50MT of edible tubers annually. The long-term storage potential of these tubers means that yams are vital for food security in developing countries. Additionally, many species are important sources of pharmaceutical precursors. Despite these attributes *Dioscorea spp.* are largely understudied in comparison to other tuberous staples such as cassava (*Manihot esculenta*) and sweet potato (*Ipomea batas*).

Despite use of leaves in traditional medicines, phytochemical analysis using metabolite profiling has not previously been conducted on the foliage of *Dioscorea* species. Foliage material is a waste product of *Dioscorea* production, yet some species generate substantial biomass each annual growth cycle. Utilising waste foliage has potential to provide a renewable source of high-value natural products, whilst sustaining conservation of species.

In this study, the polar extracts from foliage material of a diverse collection of *Dioscorea* were analysed via gas chromatography- mass spectrometry. Metabolite profiles obtained discriminate species relating to phylogenetic and morphological characteristics and provide leads for potential sources of valuable compounds.

P-5

Different content of phytosterols, tocopherols and carotenoids in two carrot root suspension cultured cells lines.

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Phytosterols, tocopherols and carotenoids are isoprenoid compounds which play an essential role in plant protection but also they are important healthful compounds for humans due to the known powerful antioxidant activity. Carotenoids and tocopherols play an important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging reactive oxygen species (ROS) and suppressing lipid peroxidation. Carotenoids also provide precursors for the biosynthesis of the plant hormone, abscisic acid (ABA) and for production of the volatile fruit flavor/aroma. These metabolites are considered to be beneficial effects on the prevention of a variety of major diseases, including certain types of cancers and eye diseases.
On the other hand, phytosterols are integral components of the plant cell membranes and responsible for its permeability and fluidity. In addition, phytosterols affect the biological function of lipid rafts in response to stress and play an important role in cellular processes as precursors for brassinosteroid biosynthesis. Moreover, phytosterols have important pharmacological activities, including cholesterol-lowering, anti-inflammatory, antitumor effects against lung, stomach, ovary and estrogen-dependent human breast cancer.
Traditionally, plant metabolites have been directly extracted from raw plant materials. A biotechnological alternative for their production is the use of plant cell cultures as cell biofactories because a large part of these metabolites may be accumulated in a higher extent comparing to their low levels found in intact plants.
In this work, we have analyzed the content of carotenoids, phytosterols and tocopherols in two different cell lines obtained from carrot roots: one photosynthetic green cell line and another highly β-carotene producer orange line. Significant differences in lutein and tocopherol content were found in the photosynthetic cell line which agrees with the plastid localization of the enzymes responsible for their biosynthesis. In addition, the effect of cyclodextrins and methyl jasmonate on the production of these metabolites was analyzed.

P-6

Potentially neuroactive amines in kiwifruits.

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The beneficial effects of a diet rich in fruits and vegetables on human health are generally recognized. The protective effect of a diet rich in fruit and vegetable on cardiovascular diseases and some kind of cancer has been shown in many investigations, including the large scale study using the data coming from the EPIC (European Prospective Investigation into Cancer and nutrition) initiative (Crowe *et al.*, 2011) and the very recent investigation of Oyebode and coworkers (2014). The earlier investigation inspired the launch of various national campaign such as “5-a-day” campaign in UK, France and Germany, the “Fruit and Veggies-more matters” in USA, and the “Go for 2+5” in Australia.

Some very recent investigation highlighted also a positive association between fruit and vegetable consumption and enhanced mood, happiness, psychological well-being feeling (White *et al.*, 2013, Carr *et al.*, 2013; Blanchflower *et al.*, 2012) and decreased depression (Tsai *et al.*, 2001).

However, similar reports referred on specific fruits or vegetables are very rare. Carr and coworkers (2013) reported a specific positive association between the consumption of two kiwifruits per day and less fatigue, more vigour and overall enhanced mood state, while Lin and coworkers (2011) found that kiwifruits seems to improve sleep onset, duration, and efficiency in adults. The precise molecule(s) responsible for these activities have not been yet identified; White and Carr speculated that the observed kiwi fruit effects could be due to the high content of vitamins (mainly vitamin C, D, E), folates, carotenoids, flavonoids, omega-3-fatty acids and micronutrients, while Lin and coworkers speculatively attributed the observed effect on vitamins, antioxidants and serotonin, which has been previously detected in this fruit.

Recently, in a project aimed to the metabolomics characterization of kiwifruits, we found that beside the presence of vitamin C and various different polyphenols, an interesting cocktail of metabolites, which potentially could be involved in the psycoactivities of this fruit, have been detected. This phytocomplex included tryptophan, tryptamine,

serotonin, N-acetyl serotonin and melatonin, i.e. the complete biosynthetic pathway for the production of phytomelatonin.
The putative gene responsible for tryptamine production in kiwifruit was identified and it was characterized by phylogenetic comparison with that of other plant species and by its heterologous expression in *Nicotiana benthamiana*.

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P-7

A glyco-alkaloid derivative accumulating on the surface of tomato plants can protect them from parasitic dodder attack

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We used the large *S. lycopersicum* x *S. pennellii* introgression line collection of tomato to screen for lines that exhibit resistance against parasitic giant dodder. Giant dodder (*Cuscuta reflexa*) is a parasitic weed that causes annual agronomic losses in the million-Euro range in many parts of the world. Currently, no pesticides are known that can efficiently limit the attack by these weeds and their spreading.

Among the resistant tomato introgression lines we found one that appears to repel attacking dodder. An analysis of the surface composition of this line as well as the parental lines revealed some unique compounds that could evoke this effect. GC, GC/MS and HPLC analyses suggest that the compound is a derivative of α-tomatine, a highly common component of tomato plants belonging to the class of glyco-alkaloids. The fact that glycol-alkaloids are wide-spread among solanaceous crop plants and have well known effects also on other pathogens makes the derivative a promising candidate for developing much needed novel remedies against parasitic plants using the natural “arsenal” of resistant lines.

P-8

Transcriptomics and metabolomics approaches towards better understanding of BIA biosynthesis in opium poppy

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Alkaloids, being member of high value plant natural products (PNPs), have several types including benzylisoquinoline alkaloids (BIA). The opium poppy (*Papaver somniferum* L.), produces a number of BIAs including morphine, codeine, noscapine, and thebaine. Numerous gene transcripts and complex regulatory pathways are involved in the biosynthesis. To better understand the molecular mechanisms behind the synthesis and to identify the responsible genes, we have applied several approaches such as gene silencing, next generation sequencing (NGS), microarray and HPLC/ToF-MS tools. Genom-wide expression analyzes were performed on four opium poppy cultivars producing high level of morphine, thebaine, and noscapine. Selected genes were silenced and overexpressed to identify their functional roles in different opium poppy tissues. Furthermore, the genetically manipulated plants were subjected to large-scale metabolomic and transcriptomic analyses. Additionally, microRNA regulation of BIA biosynthesis in opium poppy was deeply studied by NGS tools. Complex regulatory network of opium poppy miRNAs and their corresponding target transcripts were comprehensively investigated. Our studies based on transcriptomics and functional genomics present detailed analyses to gain a broader perspective on BIA biosynthesis.

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P-9

Functional roles of triterpenes

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Triterpenes are plant natural products with a vast array of structural diversity and spectrum of biological activities. They have a common biogenic origin with sterols *via* cyclization of 2,3-oxidosqualene and further modifications of the basic backbone. Due to their established pharmaceutical bioactivities, simple triterpenes have well-studied modes of action in signaling pathways in mammalian cells. We have identified the legume nodule formation as a unique system to study localized, inducible cell divisions in the plant root and use the biological resources of the model legume *Lotus japonicus* in order to identify plant targets that are subjected to the action of simple triterpene molecules,such as lupeol, during nodule organogenesis. In addition the mode-of-action of triterpenes on protein targets (type and level of effect, molecular associations) is determined.

P-10

Anthocyanin-regulating MYB and bHLH transcription factors control very diverse non-flavonoid pathways in Nicotiana benthamiana leaves.

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Coloration of plant organs such as fruit, leaves and flowers through anthocyanin production is governed by transcription factors (TFs). In this study we introduced Rosea1 (ROS1) and Delila (DEL), into *Nicotiana benthamiana* leaves by agroinfiltration. ROS1 and DEL form a pair of well-characterized TFs from Snapdragon, which specifically induce anthocyanin accumulation when expressed in tomato fruit. In *N. benthamiana*, robust induction of a single anthocyanin, delphinidin-3-rutinoside (D3R) was observed after expression of both ROS1 and DEL. Surprisingly in addition to D3R, a range of additional metabolites were also strongly and specifically up-regulated upon expression of ROS1 and DEL. Except for the D3R, these induced compounds were not derived from the flavonoid pathway. Most notable among these are nornicotine conjugates with butanoyl, hexanoyl, and octanoyl hydrophobic moieties, and phenylpropanoid-polyamine conjugates such as caffeoyl putrescine. Our study showed that the effect of ROS1 and DEL expression in *N. benthamiana* leaves extends beyond the flavonoid pathway. Apparently the same transcription factor may regulate different secondary metabolite pathways in different plant species. Currently we are identifying the gene networks that mediate synthesis of the observed metabolic changes in *Nicotiana*

P-11

The genetic bases of stilbenoids biosynthesis upon downy mildew infection in grapevine

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Downy mildew, caused by the oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is one of the major threats of grapevine cultivation particularly in warm and humid climate. All traditional grapevine cultivars (*Vitis vinifera* L.) are susceptible to downy mildew, and its control mainly relies on the use of synthetic fungicides which are costly and have environmental impact as well. Therefore, developing new varieties that are resistant to downy mildew through breeding is a promising alternative.
Stilbenoids represent the major antimicrobial phenolic compounds in grapevine and there are compelling evidences that they contribute to both constitutive and induced resistance mechanisms. As a consequence, a deep comprehension of the genetic bases of stilbenoids biosynthesis is desirable. For several years, we have been investigating the roles of the *Vitis* stilbenoids as determinants of downy mildew resistance, taking advantage of an interspecific population derived from the *Vitis* hybrid Merzling × *V. vinifera* cv Teroldego.
With this aim, 130 F1 individuals of this segregating population have been characterized at both genotypic and phenotypic level. Regarding the latter, a comprehensive analysis of leaf phenolics (including 16 different stilbenoids) upon *P. viticola* infection has been carried out. Our results indicated a significant induction of several stilbenoids following downy mildew infection in a subset of individuals characterized by a high degree of resistance. Then, QTL analysis lead to the identification of genomic regions associated to stilbenoids production. Moreover, a kinetic analysis of the accumulation of the different stilbenoids and of the expression of some genes associated to their biosynthesis has been carried out upon pathogen infection. This kinetics has revealed a

significant correlation between the type of stilbenoid and specific members of the stilbene synthase gene family.
Finally, the ongoing comparison between the stilbenoids biosynthesis results and the findings derived from a QTL analysis for downy mildew resistance conducted in parallel will putatively provide common as well as specific regions associated to both traits.

P-12

Pigmentation "clue" in Yellow Raspberries - Carotenoids and masking effect of anthocyanins

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Fruit pigmentation in raspberries (*Rubus idaeus* L.) is a complex phenomenon and one of the most important traits where a range of colour patterns from deep purple to yellow exists. Anthocyanins and carotenoids are known to be the main pigments, while colour of fruits is mainly considered to be due to varying anthocyanin contents. However, carotenoids seem to be responsible for the yellow colour and they are just masked by anthocyanins in the case of red varieties. Even though apocarotenoids, carotenoid breakdown products, are responsible for the characteristic raspberry flavour, and although these compounds seem to have very beneficial effects in health, raspberries carotenoid composition and carotenoid biosynthesis in raspberry has been very little studied.

To better understand the origin of yellow colouration observed in raspberry varieties, targeted metabolomics approaches focusing on carotenoid and (poly)phenolic pattern were performed (1,2). The composition of carotenoids, chlorophyll derivatives and tocopherols in raspberries of different varieties, including yellow and red varieties, was studied during a STSM (EC at RHUL) over different ripening stages. The profile of pigments in ripening raspberries changes drastically, with a dramatic decrease of β-carotene and chlorophyll derivatives, the xanthophyll lutein has also decreased but not to the same extent. In contrast lutein esters increased and are present in ripe raspberries esterified with saturated fatty acids with C8 to C16 chains. Ripe raspberries contain considerable amounts of lutein, lutein esters, and tocopherols (up to 20, 49 and 366 mg/kg dry weight, respectively). As a result, the different samples analysed show different contents of carotenoids and tocopherols. Whether the differences arise from the variety or other factors such as the environmental conditions needs to

be ascertained but isoprenoids should not be neglected when considering raspberry antioxidant and nutraceutical composition.

Fruit ripening in yellow raspberries is associated with biosynthesis of different amounts of carotenoids in the biosynthetic pathway. Genetic and functional analysis of the pathway genes will further elucidate the important role of carotenoid pigments in raspberry. There is also possibility of mutations in the pathway that may indicate reason for different amounts of carotenoids and various fruit color shades in yellow raspberries. For functional characterization of putative carotenoid pathway genes from *Rubus* in microbial host (STSM of MZR at RHUL) we took advantage of an in house preliminary draft of the raspberry genome to identify homologous carotenoid pathway genes and to clone candidate genes into standard expression cassettes. These plasmids were used to complement with plasmids capable of generating different carotenoid precursors. The co-transformation results in a visible colour change in the *E. coli* host, indicating thefunctional assignment. Upon the detection of visual colour, analysis of carotenoids was carried out to ascertain the carotenoids present. In the case of carotenoid cleavage enzymes the visible screen is the reduction in colour compared to the precursor line.
Based on these results a pathway map for pigmentation in raspberry will be predicted and utilized for biotechnological production of specific carotenoids and aroma compounds.

Session II

Filling gaps in plant secondary metabolism and metabolic engineering

Key-note lecture

Discovery of aromatic prenyltransferase gene family and its application to metabolic engeering

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In phytochemistry field, many prenylated phenolic compounds have been isolated from a variety of medicinal plants as biologically active substances. Prenylated core compounds are mostly flavonoids, coumarins, and other aromatic compounds like phloroglucinols, xanthones, and simple phenylpropanoids. Occurrence of prenylated aromatics is wide, while there is a chemotaxonomic tendency that those compounds are frequently found in Leguminosae, Moraceae, Apiaceae, and Rutaceae. The contribution of prenyl residue to increase biological acitivities, in general, draw a large attention of researchers working on biosynthesis of secondary metabolites, whereas the identification of prenyltransferase recognizing those phenolic compounds was difficult due to the membrane-bound properties of this enzyme family.

We have identified the first flavonoid-specific prenyltransferase N8DT-1 from a medicinal plant *Sophora flavescense* (Leguminosae), which was followed by isolation of various flavonoid prenyltransferases, and a hop prenyltransferase involved in bitter acid biosynthesis as well. Recently, coumarin-specific enzymes have been identified from parsley and lemon, which enabled us to overview, from which enzyme family prenyltransferases for phenolic compounds have been evolved, and how much sequence similarity reflects to the enzymatic functions, such as substrate specificity.

As an example of metabolic engineering using such a prenyltransferase, we introduced coumarin-specific prenyltransferase PcPT into *Nicotiana benthamiana*, accompanied with 4–coumaroyl CoA 2'–hydroxylase involved in the coumarin ring formation from 4–coumaroyl CoA, a common plant metabolite. While wild type *N. benthamiana* does not produce coumarin, the double transformant revealed clear production of dimethylsuberosin, suggesting that furanocoumarin pathway may be reconstructed in coumarin-non-producing host plants. In the Conference, limitations of metabolic engineering using prenyltransferase genes are also discussed.

O-5

Enhanced sequestering of sesquiterpenes by coexpression of pathway genes with ABC-transporter and LTP genes in *Nicotiana benthamiana*

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We have characterized *Artemisia annua* genes of the artimisinin biosynthesis pathway by transient reconstitution of the entire biosynthesis pathway in *N.benthamiana*. This results in high level of glycosylated artemisini acid (AA) and dehydroartemisinic acid (DHAA), while only very low levels of free AA and DHAA and no artemisinin is detected at six days post-agroinfiltration. It has been postulated that in *A.annua* AA and DHAA are sequestered to the apoplastic space of glandular trichomes, where they are photochemically converted to arteanuin B and artemisinin, respectively. This transfer of AA and DHAA to the apoplast may involve membrane transporters. In addition, there could be a role for Lipid Transfer Proteins (LTP) as well as these are expressed at high levels in *A. annua* glandular trichomes. We tested the effect on free levels of AA and DHAA of two PDR type membrane transporters and three LTPs from *A. annua* in combination with the artemisinin pathway genes by transient expression in *N.benthamiana*. The pathway+PDR genes and pathway+LTP genes showed little or no effect on AA and DHAA levels, but the combination of pathway+PDR+LTP genes showed a 5-fold increase in AA and DHAA in total leaf extracts at 6 days post agroinfiltration. Test of the individual PDR1/2 with individual LTP1/2/3 showed highest activity for the combination PDR2+LTP3 indicating specificity both at the level of PDR and LTP. The higher level of free AA and DHAA suggests that these compounds are sequestered away from the cytosol where they are subject to glycosylation. Indeed, a wash of the apoplast of leaves at 5 days post-agroinfiltration showed the presence of AA and DHAA, while no glycosylated AA/DHAA was present in the apoplast wash. AA and DHAA were also detected in the apoplast of leaves agroinfiltrated with only the pathway genes, suggesting a very low intrinsic transport activity for these compounds in *N. benthamiana*. AA and DHAA in the apoplast wash was increased up to 3-fold when pathway genes were combined with PDR+LTP. However, AA and DHAA in the apoplast

wash represented only 1% of the total AA and DHAA present in a leaf. This means either that the apoplast wash is not very efficient or that the remains of AA and DHAA are sequestered in intracellular vesicles that may be loaded by the combined action of PDR2+LTP3. Localisation studies of the LTP-GFP fusion proteinsshow both intracellular labeling of the ER and extracellular labeling, including LTP filled bridges between cells of the spongy mesophyll. The fluorescence signal from the LTP-GFP was quantified and we could show that the total fluorescence level of LTP-GFP did not increase when coexpressed with PDR but did increased by 20% when coexpressed with the PDR + pathway genes. This suggests that pathway gene dependent active transport by the PDR somehow results in stabilisation of the LTP-GFP fusion protein. In these experiments there was no transcriptional difference in 35S-LTP-GFP transcript levels.

O-6

A molecular toolbox to engineer the metabolic flux of the anticancer alkaloids from *Catharanthus roseus*

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The medicinal plant *Catharanthus roseus* accumulates in the leaves the anticancer terpenoid indole alkaloids (TIAs) vinblastine and vincristine, universally known as the Vinca alkaloids. These TIAs were the first natural anticancer products to be clinically used, and are still among the most valuable agents used in cancer chemotherapy. Hence, the TIA pathway has been intensively investigated and *C. roseus* has become one of the most studied medicinal plants. The TIA pathway is highly complex, involving more than 30 steps, and it presents subcellular and multi-cellular compartmentation in *C. roseus* leaves, predicting several transmembrane transport events. Although much is known about the biosynthesis and regulation of TIAs, gene/enzyme

characterization is still lacking for the bottleneck biosynthetic steps, the membrane transport mechanisms of TIAs are poorly characterized, and no effective master switches of the pathway have been identified. Here, we will present our recent and ongoing efforts to identify the key genes involved in the biosynthesis, transport and regulation of TIAs, aiming to generate a molecular toolbox enabling the design of successful metabolic engineering strategies for increased levels of the *C. roseus* anticancer alkaloids.

O-7

Multiple copies of a simple MYB-binding site confer trans-regulation by flavonoid-related R2R3 MYBs

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Recently there has been considerable research activity into anthocyanins and their contribution to a healthy diet. In apple, the R2R3 MYB transcription factor, MYB10, controls the accumulation of anthocyanin. We have genetically engineered apple with MYB10 to alter various flavonoid subclasses, particularly anthocyanins. These apples have been tested in both consumer trials and used in animal health studies where they associated with decreases in inflammation markers and changes in gut microbiota. MYB10 is also able to activate its own expression by binding its own promoter at a specific motif; the R1 motif. In red-fleshed apple germplasm this motif is mutated and comprises a further five tandem repeats of R1 to form a minisatellite repeat unit, R6. This modification results in a gain of function, producing an increase in anthocyanins throughout the plant and a striking phenotype that includes red foliage and red fruit flesh. We have used this mutation to show that other anthocyanin-related R2R3 MYBs from pear, strawberry, petunia, kiwifruit, and Arabidopsis are also able to up-regulate promoters containing the R6 motif. To examine specificity of this motif we screened other members of the R2R3 MYB super-family against promoters harbouring the R6 mutation. Only MYBs from sub-groups 5, 6, and 7 activate the R6 motif. Insertion of the R6 motif into orthologous promoters of pear (*PcMYB10*) and *Arabidopsis* (*PAP1*) resulted in auto-regulation of the MYB and plants containing this construct showed elevated anthocyanin levels. Introduction of the R6 motif into the promoter region of an anthocyanin biosynthetic gene, the cytochrome P450 enzyme, F3'5'H, altered the resulting anthocyanic profile, producing elevated levels of delphinidin in both tobacco and kiwifruit. This naturally occurring motif

provides a versatile tool to re-engineer novel MYB-regulated responses across a range of plant species.

O-8

Plant roots as a perfume? Overexpression of a MYB transcription factor leads to methyl anthranilate emission in *M. truncatula* hairy roots

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Plants respond to herbivores or pathogen attack by activating specific defense programs that include the production of bioactive secondary metabolites to eliminate or deter the attackers. This process is regulated by a signaling cascade that involves jasmonates, ubiquitous oxylipin-derived phytohormones that also play essential roles in the regulation of many developmental and growth processes. Through transcript profiling of jasmonate-treated *M. truncatula* suspension cells we identified a MYB transcription factor that, when expressed in *M. truncatula* hairy roots, leads to the emission of the volatile compound methyl anthranilate, giving the roots a fruity scent. RNA-Seq analysis of the fragrant roots revealed the upregulation of a methyltransferase that was subsequently characterized as an anthranilate *O*-methyltransferase. Activation assays in tobacco protoplasts, however, revealed that the anthranilate *O*-methyltransferase promoter was not the direct target of the MYB transcription factor. Further probing of the RNA-Seq data identified a PLATZ repressor protein, the promoter of which is bound and activated by the MYB transcription factor. Through promoter deletion and mutation analysis we identified an 8 bp motif in the promoter of the PLATZ repressor that is essential for binding of the MYB transcription factor and subsequent promoter activation. Expression of the PLATZ repressor in transgenic *M. truncatula* hairy roots led to transcriptional silencing of the MYB transcription factor, indicating that this

transcription factor possibly activates a negative feedback loop, a principle that seems to be widespread in plant secondary metabolism and is likely in place to safeguard plant integrity and protect the plant from overaccumulation of particular bioactive specialized metabolites.

O-9

Driving metabolic flux towards high content of abietane-type diterpenes in *Salvia sclarea* hairy roots

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Abietane diterpenoids (*e.g.* aethiopinone, 1-oxoaethiopinone, salvipisone, and ferruginol), synthesized in the roots of different *Salvia* species, have a great medicinal value for a variety of known biological activities. In particular, aethiopinone has a promising citotoxic activity against different human tumor cell lines [1,2]. The accumulation at low levels and the limited possibility to synthesize chemically these products, prompted us to optimize their production by targeting genes of the MEP-derived pathway, from which they derive.
Elicitation studies with methyl-jasmonate and coronatine have indicated that the induced-expression of genes acting up-stream [1-Deoxy-D-Xylulose-5-Phosphate Synthase **(***DXS***)** and 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase **(***DXR*)] or more downstream [geranylgeranyl-diphosphate synthase (*GGPPS*) and copalyl-diphosphate synthase (*CPPS*)] of this pathway correlates with high-level of abietane-type diterpenes in *S. sclarea* hairy roots. Here, we report our current efforts to direct the metabolic flux towards this interesting class of compounds in *Salvia sclarea* hairy roots by tuning the expression of genes controlling putative rate-limiting steps (DXS, DXR, GGPPS, CPPS, alone or in combination), silencing of a gene acting at a lateral competitive route [*Ent*-copalyl-diphosphate synthase (Ent-CPPS)] or by over-expression of transcription factors that regulate coordinately several genes of the pathway (WRKY and MYC2).
Overall, these complementary approaches successfully enhanced the content of this class of compounds in engineered hairy roots (from 2- up to 8-fold higher than the content in the control line), paving the way to a rational design of a production platform to yield reliable amounts for a deeper understanding of their molecular targets and potential future commercialization.

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O-10

Transcriptomics and metabolomics approaches towards better understanding of BIA biosynthesis in opium poppy

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Alkaloids, being member of high value plant natural products (PNPs), have several types including benzylisoquinoline alkaloids (BIA). The opium poppy (*Papaver somniferum* L.), produces a number of BIAs including morphine, codeine, noscapine, and thebaine. Numerous gene transcripts and complex regulatory pathways are involved in the biosynthesis. To better understand the molecular mechanisms behind the synthesis and to identify the responsible genes, we have applied several approaches such as gene silencing, next generation sequencing (NGS), microarray and HPLC/ToF-MS tools. Genom-wide expression analyzes were performed on four opium poppy cultivars producing high level of morphine, thebaine, and noscapine. Selected genes were silenced and overexpressed to identify their functional roles in different opium poppy tissues. Furthermore, the genetically manipulated plants were subjected to large-scale metabolomic and transcriptomic analyses. Additionally, microRNA regulation of BIA biosynthesis in opium poppy was deeply studied by NGS tools. Complex regulatory network of opium poppy miRNAs and their corresponding target transcripts were comprehensively investigated.
Our studies based on transcriptomics and functional genomics present detailed analyses to gain a broader perspective on BIA biosynthesis.

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O-11

Transcript profiling of jasmonate-elicited *Taxus* cells to identify new genes involved in taxane biosynthesis: a new gene encoding a β-phenylalanine-CoA ligase.

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The number of studies using "omics" approaches is growing, since these technologies allow the generation of vast amounts of data at different levels of plant biology, ranging from metabolite patterns to gene sequences and protein expression. Using this information, we are able to integrate cellular networks in plant systems and try to understand how the whole cell works; it can also lead to the discovery of unknown steps in specific biosynthetic pathways.

Plant cell cultures constitute eco-friendly biotechnological platforms for the production of plant secondary metabolites with pharmacological activities, as well as a suitable system for extending our knowledge of secondary metabolism. Taxol and related taxanes are high-value secondary metabolites produced by several *Taxus* species. Due to their antineoplastic activity, they have been widely used in cancer therapy. To date, several biosynthetic steps of these important compounds remain undefined. In the present work, a genome-wide expression analysis of jasmonate-elicited *Taxus baccata* cell cultures by complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) indicated a correlation between an extensive elicitor-induced genetic reprogramming and increased taxane production in the targeted cultures. Subsequent *in silico* analysis allowed us to identify 15 genes with jasmonate-induced differential expression as putative candidates for genes/enzymes involved in five unknown steps of taxane biosynthesis. Among them, the *TB768* gene showed a strong homology and a predicted-3D structure very similar to other genes

previously reported to encode acyl-CoA ligases, suggesting a role in the formation of the taxol lateral chain. *In vitro* analysis showed that the *TB768* gene encodes an acyl-CoA ligase localized in the cytoplasm able to convert β-phenylalanine into its derivative CoA ester. The latter is then attached to baccatin III in one of the last steps of the taxol biosynthetic pathway.

As well as confirming that the *TB768* gene encodes a cytosolic CoA ligase with an important role in the final part of the taxol biosynthetic pathway, our work endorses that transcriptomic profiling monitored by cDNA-AFLP, combined with bioinformatics analysis, constitutes a cutting-edge set of tools for the identification of new genes involved in the metabolism of valuable bioactive compounds. The identification of this gene will contribute to the establishment of sustainable taxol production systems through metabolic engineering or synthetic biology approaches.

O-12

Detection of wild alleles to engineer ascorbic acid metabolic pathway in tomato

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Solanum lycopersicum represents an important source of antioxidants and other bioactive compounds associated with a reduced risk of cancer, inflammation and cardiovascular diseases. In particular, ascorbic acid (AsA) protects against oxidation of LDL (low-density lipoprotein) in vascular endothelial cells. In tomato, AsA is highly bioavailable, thus a regular consumption of tomatoes can increase cell protection from DNA damage induced by oxidant species. The increase of ascorbic acid content of tomato fruit constitutes one important objectives of tomato breeding and a metabolic engineering approach could help reach this objective. Up to date the annotation of the biosynthetic pathways of ascorbic acid in tomato is still ambiguous. Therefore in our laboratory a complete dissection of the Ascorbic Acid (AsA) pathway in tomato was performed in order to identify the putative loci expected to contribute to the entire pathway, their genome distribution and their possible redundancy in terms of multi-copy genes. This was done through the integration of transcriptomic data with genome-scale resources that allowed us to investigate in depth, through a bioinformatic approach, the genes involved in AsA metabolism. In particular, a reliable reference query from sequences UniProtKB/Swiss-Prot database was used to identify 238 genes associated with the 42 enzymatic reactions reconstructed from the AsA metabolism. Data coming from the bioinformatic analyses were used to identify genes and alleles controlling the higher AsA content in a *Solanum pennellii* introgression line (IL 7-3) previously selected in our laboratory. We performed a metabolic analysis of tomato fruits at different ripening stages from the introgression line IL 7-3 and of the control cultivated genotype M82. These analyses revealed a higher content of ascorbic acid in IL 7-3 fruits compared to M82 in all the tested ripening stages. No genes of the main biosynthetic pathway were mapped in the wild genomic region of 25 cM, however several genes of alternative biosynthetic and transport pathways were found such as one polygalactorunase and one Nucleobase Ascorbate Transporter. Variants were detected for these genes between the wild

and the cultivated alleles. Their role in increasing Asa levels will be further investigated.
In the future the favorable alleles detected in this study could be transferred into cultivated background through breeding or metabolic engineering.

O-13

Polyketide synthases responsible for 4-hydroxy-5-methylcoumarin biosynthesis in gerbera

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Gerbera (*Gerbera hybrida*) is an economically important ornamental species and a model plant of the Asteraceae family in flower development and secondary metabolism research. Gerberin and parasorboside, two bitter tasting glucosidic lactones, are produced in high amounts in nearly all gerbera tissues. Gerbera and its close relatives also produce a rare coumarin, 4-hydroxy-5-methylcoumarin (HMC). Unlike most coumarins, 5-methylcoumarins have been suggested to be derived through the acetate-malonate pathway. All of these polyketide derived glucosides are considered to have a role in defense against herbivores and phytopathogens in gerbera.
Gerbera expresses three genes encoding 2-pyrone synthases (*G2PS1-3*). The enzymes are chalcone synthase -like polyketide synthases with altered starter substrate specificity. We previously showed that G2PS1 is responsible for the synthesis of triacetolactone (6-methyl-4-hydroxy-2-pyrone), a putative precursor of gerberin and parasorboside. Here we show that polyketide synthases G2PS2 and G2PS3 are responsible for the biosynthesis of HMC in gerbera. *G2PS2* is expressed in the leaf blade and inflorescences of gerbera, while *G2PS3* is strictly root specific. Heterologous expression of *G2PS2* or *G2PS3* in tobacco leads to formation of 4,7-dihydroxy-5-methylcoumarin, apparently an unreduced precursor of HMC, while ectopic expression in gerbera leads to HMC formation in tissues where control plants do not express the genes or accumulate the compound.

O-14

Type III polyketide synthases from poison hemlock

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Hemlock alkaloids are notorious for their toxicity exemplified by the killing of Sokrates in ancient Greece. Coniine is representative for a group of piperidine alkaloids that occur only in a few non-related genera of plants i.e. *Conium* (Apiaceae), *Sarracenia* (Sarraceniaceae) and *Aloe* (Asphodelaceae). Early labelling experiments in *Conium* revealed that these alkaloids are biosynthetically derived from acetate units via a polyketide rather than directly from amino acids as shown for other piperidines. These results point to the involvement of a type III polyketide synthase to facilitate condensation of an acetyl CoA and three malonyl CoA units to form a tetraketide. The formation of the established 5-ketooctanal intermediate would further involve reduction and incorporation into gamma-coniceine together with alanine in an established transaminase catalyzed reaction. A further reduction step finally delivers coniine.

Here we describe the isolation and characterization of several candidate genes for PKSs responsible for coniine formation in *Conium maculatum*. The genes were cloned, heterologous expressed in *E. coli*, purified and investigated by precursor feeding studies. The biochemical characterization of the recombinant PKSs provides evidence for their involvement in different metabolic pathways.

O-15

Monoterpenol oxidative metabolism for the biosynthesis of bioactive compound

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Analysis of gene co-expression and functional analysis in *Arabidopsis thaliana* identified CYP76C enzymes as involved in floral monoterpenoid metabolism. Functional screening revealed that different enzymes metabolized monoterpenols and at least three of them led to the production of different linalool oxides in arabidopsis flowers. CYP76C1 appeared as the major player and sequentially metabolized linalool and its oxidized derivatives leading to the production of lilac compounds and 8-COOH-linalool *in vitro* and *in vivo*. The role and ecological function of CYP76C1 and the linalool oxides derivatives were investigated on antagonistic *Arabidopsis* flower visitors. Choice test experiments revealed that all the studied insects were shown to prefer the flowers of the *cyp76c1* mutants over the wild-type flowers. Our ecological studies thus suggest that CYP76C1 and resulting products are involved in plant defence against flower visitor and florivore insects.

This work provides new tools for the production of fragrant and aromatic compounds and for engineered production of biopesticides and insect deterrents. It will be put into perspective with our recent demonstration of the involvement of CYP76 enzymes in multiple geraniol oxydations and in the biosynthesis of (seco)iridoids and terpenoid indole alkaloids in *Catharantus roseus* and of the formation of terpenic aroma in wine and fruits.

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O-16

TILLING analysis of a *Medicago truncatula* mutant collection for identification of CYP450s involved in triterpene saponin biosynthesis

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Triterpenic saponins are bioactive secondary metabolites synthesized by many plant families among which legumes (Fabaceae). The exploitation of saponins as pharmaceutics, agrochemicals and in food and cosmetic industries has raised interest in unravelling saponin synthesis in plants to identify the enzymes involved in this pathway. In the *Medicago* genus, saponin are a complex mixture of triterpene glycosides derived from the isoprenoid pathway via the cyclization of 2,3-oxidosqualene to give the β-amyrin nucleus. Oxidative modifications mediated by cytochromes P450 (CYP450s) produce the aglycone moieties (sapogenins) that are successively glycosylated by glycosyltransferases (GTs) to give saponins.
We report the identification of a CYP450 gene involved in sapogenin biosynthetic pathway by a reverse genetic TILLING approach in a *M. truncatula* ethylmethanesulfonate (EMS) mutagenized collection (Porceddu et al., 2008; Carelli et al., 2013). The functional characterization of this gene by expression in an in vitro yeast system allowed to define its role in sapogenin pathway, filling in a gap in β-amyrin oxidative modifications generating the triterpenic aglycones naturally found in *M. truncatula* and *M. sativa* (alfalfa), one of the major forage crop worldwide. The affinity of this CYP450 for substrates with different substitutions at multiple carbon positions is investigated in the same in vitro yeast system in order to characterize its catalytic features. The relationships with other known CYP450s involved in the same pathway are also studied. This information can be used for the breeding/engineering of 'natural' triterpene biosynthesis in plants as well as for 'synthetic' biosynthesis in heterologous hosts or in cell-free systems.

Session II Posters

Filling gaps in plant secondary metabolism and metabolic engineering

P-13

Natural raspberry ketone production via bioconversion using plant cell cultures

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A characteristic aroma component in raspberry (*Rubus idaeus*) fruits is 4-(4-hydroxyphenyl)butan-2-one, also called raspberry ketone or frambinone. Natural raspberry ketone flavour ranks second behind natural vanillin, with the total potential market value between 6 to 10 million euros, although currently it is not commercially available. The biosynthesis of this industrially demanded flavour compound is comparatively well characterised diketide pathway, involving the condensation of 4-coumaryl-CoA and malonyl-CoA. First coumaroyl-CoA and malonyl-CoA form p-hydroxybenzalacetone (4-OHBA) in a decarboxylative condensation catalysed by benzalacetone synthase (BAS). An NADPH-dependent reductase from raspberry, called raspberry ketone/zingerone synthase 1 (*Ri*ZS1), has been suggested to be responsible for the last step in raspberry ketone biosynthesis. However, this gene has not been functionally tested in planta so far.

Raspberry ketone has been successfully produced by bioconversion with feeding either 4-OHBA or betuligenol (rhododendrol). The aim of this work was to perform functional testing of *Ri*ZS1 gene by heterologous expression in tobacco. In addition, we present the bioconversion studies related to raspberry ketone performed with plant cell cultures and present the hypothesis on the roles of different raspberry ketone biosynthetic genes.

P-14

Searching for transcription factors putatively regulating rate-limiting steps of anticancer alkaloid biosynthesis and accumulation in *Catharanthus roseus*

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Catharanthus roseus accumulates in low levels the terpenoid indole alkaloids (TIAs) vinblastine (VLB) and vincristine (VCR), two valuable agents used in cancer chemotherapy. TIA biosynthesis is organ specific, with VLB and VCR being produced only in leaves, and it shows multi-cellular compartmentation, since early steps occur in leaf epidermis, while late steps occur in specialized laticifer and idioblast cells - possibly the single sites in the plant where the biosynthesis of VLB and VCR is completed. Induction by methyl jasmonate is also a hallmark of TIA metabolism. Although much is known about the biosynthesis and regulation of TIAs, *C. roseus* transcription factors (CrTFs) up-regulating the late, bottleneck part of the pathway were never identified, and research efforts to improve the production of VLB and VCR have remained unsuccessful. Recently, we implemented a targeted omic strategy involving the isolation of the idioblast leaf cells specialized in the late bottleneck steps of TIA biosynthesis, followed by

their differential transcript profiling. This strategy enabled the identification of several candidate CrTFs that are significantly up-regulated in idioblasts and are thus strong candidates for the regulation of the late bottleneck TIA pathway occurring in these cells. Here, one CrTF isolated by this strategy, together with CrTFs previously shown to induce the TIA early pathway, and AtTFs involved in the methyl jasmonate response in *Arabidopsis thaliana,* were transiently overexpressed alone or in selected combinations, in *C. roseus* leaf protoplasts, in order to investigate their potential as molecular tools for engineering the improved production of the *C. roseus* anticancer alkaloids.

P-15

Optimization of strictosidine production by *Catharanthus roseus* cell cultures

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Catharanthus roseus is an intensively studied medicinal plant due to the presence of terpenoid indole alkaloids (TIAs) some of which possess important medicinal value in treating cancer and hypertension. As the natural production of those valuable compounds in the plant is limited, scientists worldwide put forward tremendous efforts over the past three decades to gain further understanding of the TIA biosynthesis and its regulatory mechanisms with the aim to realize increased productivity in plants and/or in cell- and hairy root cultures.
Even though major parts of the biosynthesis pathway have now been elucidated in detail on both the chemical and molecular level, the problems to control TIA biosynthesis and to achieve high level accumulation have not been solved yet.

During an initial screening of different *C. roseus* cell lines developed at Leiden University, we found one particular cell line that accumulated strictosidine, which is the primary alkaloid precursor of the TIAs in *Catharanthus.* This cell line was further investigated with the aim to optimize strictosidine production and to find the triggers to enhance conversion to down-stream TIAs.

Here we present the effect of cultivation cycle, culture volume and feeding & elicitation strategies on precursor, strictosidine and TIA accumulation in this cell line.
In addition, qPCR analysis of early genes in strictosidine biosynthesis will be presented revealing typical differences in expression levels and in the responses to feeding & elicitation between a non-producing and the strictosidine producing cell line.

P-16

TILLING analysis of a *Medicago truncatula* mutant collection for identification of CYP450s involved in triterpene saponin biosynthesis

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Triterpenic saponins are bioactive secondary metabolites synthesized by many plant families among which legumes (Fabaceae). The exploitation of saponins as pharmaceutics, agrochemicals and in food and cosmetic industries has raised interest in unravelling saponin synthesis in plants to identify the enzymes involved in this pathway. In the *Medicago* genus, saponin are a complex mixture of triterpene glycosides derived from the isoprenoid pathway via the cyclization of 2,3-oxidosqualene to give the β-amyrin nucleus. Oxidative modifications mediated by cytochromes P450 (CYP450s) produce the aglycone moieties (sapogenins) that are successively glycosylated by glycosyltransferases (GTs) to give saponins.
We report the identification of a CYP450 gene involved in sapogenin biosynthetic pathway by a reverse genetic TILLING approach in a *M. truncatula* ethylmethanesulfonate (EMS) mutagenized collection (Porceddu et al., 2008; Carelli et al., 2013). The functional characterization of this gene by expression in an in vitro yeast system allowed to define its role in sapogenin pathway, filling in a gap in β-amyrin oxidative modifications generating the triterpenic aglycones naturally found in *M. truncatula* and *M. sativa* (alfalfa), one of the major forage crop worldwide. The affinity of this CYP450 for substrates with different substitutions at multiple carbon positions is investigated in the same in vitro yeast system in order to characterize its catalytic features. The relationships with other known CYP450s involved in the same pathway are also studied. This information can be used for the breeding/engineering of 'natural' triterpene biosynthesis in plants as well as for 'synthetic' biosynthesis in heterologous hosts or in cell-free systems.

P-17

Chemical and transcriptomic analyses reveal novel steps in labdane-type diterpenes in trichomes of *Cistus creticus*

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Cistus creticus, a species endemic in the Cretan flora, produces large amounts of the major terpene types, monoterpenes, sesquiterpenes and diterpenes. Major constituents of this plant are labdane-type diterpenes, notably manoyl oxide, 13-epi-manoyl oxide, 3β- acetoxy-13-epi-manoyl oxide, sclareol, labda-7,13(E)-dien-15-ol, labda-7,13(E)-dien-15-yl acetate, labda-13(E)-ene-8α,15-diol and labda-13(E)-ene-8α-ol-15-yl acetate. The biosynthesis of these compounds takes place in the plant's glandular trichomes. Labdane-type diterpenes are highly bioactive and exert several pharmacological properties, acting as antibacterial, cytotoxic and anticancer agents. Previously, we have functionally characterized a number of terpene synthases including copal-8-ol diphosphate synthase (CcCLS) the first step in the biosynthesis of several labdane type diterpenes. Detailed chemical analyses of fruit, leaves and isolated trichomes corresponding to four developmental stages, confirmed the young trichomes as the richest site of labdane-type diterpenes production. RNA-seq in trichomes from these young leaves revealed a number of contigs showing similarities with type II and type I diterpenes synthases and acetyl transferases. Among those, we have recently functionally characterized in *E. coli, in* yeast and in *Nicotiana benthamiana* a type II gene that transforms GGDP to labd-7,13-dien-15-yl diphosphate. Additionally, a trichome specific protein acetylates labda-13-en-8α,15-diol to 15-diol to labda-13(E)-ene-8α-ol-15-yl acetate. The above data will be discussed in the context of the biosynthetic pathway of labdane-type diterpenes in the trichomes of *Cistus creticus*.

P-18

Flavonoids pathway engineering for the induction of novel sets of healthy phytochemicals in tomato fruit

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Flavonoids are a large family of plant polyphenolic secondary metabolites. Although they are widespread throughout the plant kingdom, some flavonoid classes are specific for only a few plant species. Due to their presumed health benefits there is growing interest in the development of food crops with tailor-made levels and composition of flavonoids, designed to exert an optimal biological effect. In order to explore the possibilities of flavonoid engineering in tomato fruits, we have targeted this pathway towards novel classes of potentially healthy flavonoids. Using a structural flavonoid gene (encoding a grape stilbene synthase), we were able to produce transgenic tomatoes accumulating new stilbene-related phytochemicals. Afterwards, we performed a breeding programme taking advantage of the ability of some transcription factors (MYB12, Delila and Rosea) to strongly activate the expression of several phenylpropanoid biosynthetic genes and provide high rates of metabolic flux for different classes of polyphenols.
Biochemical analyses showed that these new tomato lines were able to accumulate high levels of specific classes of polyphenols such as stilbenes, flavonols and anthocyanins. We demonstrated that, due to the presence of the novel phytochemicals, the transgenic tomato fruits displayed a significantly higher antioxidant profile. Our data show that a combination of biosynthetic and regulatory genes together with the availability of natural tomato varieties could provide novel insights into genetic and biochemical regulation of the flavonoid pathway in this worldwide important vegetable.

P-19

Silencing of *Ent*-copalyl-diphosphate synthase gene enhances the content of bioactive abietane-type diterpenes in *Salvia sclarea* hairy roots

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There is a great demand of novel molecules to treat melanoma, the most aggressive form of skin cancer, since advanced stages are inevitably resistant to conventional therapeutic agents. We have shown recently that aethiopinone, an abietane quinone-type tricyclic diterpene, isolated from *S. sclarea* roots, but also in other *Salvia* species, is cytotoxic against human melanoma A375 cell line at a concentration which was not toxic for not tumor cells [1]. However, this compound accumulates in *S. sclarea* roots at very low concentrations (less than 0.5% DW), which constitutes a major hurdle for a deeper understanding of its molecular targets.

Abietane diterpenes are synthesized in the plastid from GGPP (geranylgeranyl-diphospate), the common precursor of several isoprenoids, such as carotenoids, chlorophyll and gibberellins. In particular, the branch point from GGPP to gibberellins is enzymatically controlled by the *Ent*-copalyl-diphosphate synthase (Ent-CPPS), a class II diterpene synthase. To increase the flux towards abietane diterpenes, we have blocked this lateral competing route by chemical inhibition of the enzymatic activity of Ent-CPPS with CCC (chlorocholine chloride), a known growth retardant, or by RNAi-mediated silencing of this gene in *S. sclarea* hairy roots. Both approaches triggered a significant increase in total abietane diterpenes diterpenes (>4-fold), without causing any growth impairment compared to control empty vector hairy roots. Target HPLC quantitative analysis revealed that the content of all the analyzed abietane diterpenes increased, with major effects on the content of salvipisone and aethiopinone.

The high biomass coupled to increased content of abietane diterpenes achieved by silencing of *Ent*CPPS gene opens the way to the extraction of suitable quantities for a more accurate molecular and pharmacological characterization of this class of compounds and, ultimately, adds additional information to developing a hairy root

platform for their potential commercialization as novel plant-derived anti-tumor molecules.

[1] Vaccaro et al, 2014. *Plant Cell Tiss Organ Culture* 119:65-77

P-20

High-level of bioactive abietane diterpenes in *S. sclarea* hairy roots by overexpression of *Ss*GGPPS and/or *Ss*CPPS genes

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We have shown recently that aethiopinone, an abietane quinone-type tricyclic diterpene, isolated from *S. sclarea* roots, but also from other *Salvia* species, has cytotoxic activity (IC_{50}=11.5 μM) against human melanoma A375 cell line at a concentration which was not toxic to not tumor cells [1]. However, this compound accumulates in *S. sclarea* roots at very low concentrations (less than 0.5% DW), which is a major hurdle for a deeper understanding of its molecular targets. We have also proved previously that higher content of abietane-type diterpenes (up to 2-3-fold) can be obtained by overxpressing *At*DXS or *At*DXR genes in *S. sclarea* hairy roots [1].
Here, we report that further accumulation of this class of compounds is stimulated by expressing alone or in combination the genes encoding geranylgeranyl-diphosphate synthase (*Ss*GGPPS), which yields GGPP, the common precursor for a large set of different plastidial isoprenoids, and copalyl-diphosphate synthase (*Ss*CPPS), the first committed enzyme of the lateral route from GGPP to abietane diterpenes.

Compared to the empty vector control line, SsGPPS/CPPS co-overexpression enhanced significantly the amount of abietane diterpenes (4-fold increase), without impairing hairy root growth rate. However, a 12-fold increase in abietane diterpenes, coupled to negligible negative effect on hairy root growth, was obtained by overexpressing *Ss*GGPPS or *Ss*CPPS alone, pointing at these enzymes as one limiting step of biosynthesis of this class of compounds. These data constitute an informative step forward in the rationale designing of a production platform for large-scale extraction of bioactive abietane-type diterpenes based on engineered *S. sclarea* hairy roots.

[1] Vaccaro et al, 2014. *Plant Cell Tiss Organ Culture* 119:65-77

P-21

Phenylpropanoid compounds in *S. melongena*: their biosynthesis and regulation

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Delphinidin-3-rutinoside (D3R), rutin and chlorogenic acid (CGA) are the major phenolic metabolites in eggplant tissues, with CGA constituting over 90% of total phenylpropanoids, and confer high nutritional value to eggplant fruits. Biochemical analysis by LC-MS was used to characterize the distribution of phenylpropanoids in different tissues (young and mature leaf, stem, root, flower, fruit skin and flesh) of Violetta Campana eggplant variety. The anthocyanin D3R was detected only in the coloured tissues (flower and fruit peel), the flavonoid rutin in all the tissues beside fruit flesh and roots, whereas a high content of CGA was found in all the epigeous tissues. Expression of key genes *PAL (phenylalanine ammonia lyase)*, *HQT (hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase)*, *DFR (dihydroflavonol reductase)* and *ANS* (*anthocyanidin synthase*) mainly involved in the biosynthesis of these compounds was evaluated by qRT-PCR to check a possible relationship between transcript accumulation and content of analysed compounds. Consistently with the high accumulation of D3R (10 ug/mg dry weight) in fruit peel and CGA (about 40 ug/mg dry weight) in fruit flesh, a higher expression of biosynthetic genes involved in anthocyanin and CGA biosynthesis was found in these tissues. Namely a significant increase (more than 100 fold) of *PAL*, *HQT*, *DFR* and *ANS* expression was detected in fruits flesh or skin compared to the other tissues, so suggesting a high biosynthetic activity for eggplant fruits.
These metabolic and molecular results prompted us to use fruit flesh tissue for the isolation of *PAL* and *HQT*coding sequences. By RACE strategy, we isolated a complete open reading frame (ORF) of 2172 bps for PAL, which encodes 720 amino acid residues, and an ORF of 1284 bps for HQT, encoding for a 430 aa protein.

To gain insight in the CGA and anthocyanins biosynthesis regulation, the promoter regions of *PAL*, *HQT* and *ANS* genes were isolated by Genome walking strategy and confirmed by BLAST search in the draft eggplant genome (Hirakawa H. et al., 2014, DNA Research doi:10.1093/dnares/dsu027). In *silico* analysis of 1.2Kb promoter regions, identified several binding motifs for Myb TFs, suggesting a major involvement of these transcription factors in the transcriptional regulation of phenylpropanoids in eggplant. Therefore, we analysed the expression of two eggplant MYB genes, proposed as anthocyanin pathway specific regulators, namely *SmAN1* and *SmMYB1*. We found *SmAN1* expression barely detectable and only in fruits, while interestingly *SmMyb1*transcripts were found in all the tissues. A putative role of *SmMyb1* also in CGA biosynthesis regulation is being investigated by functional assays in tobacco.

P-22

The oversexpression of the grape *MYBPA1* gene triggers severe changes in the phenylpropanoid pathway in tobacco flowers

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Condensed tannins (CT) are minor compounds, originating from the flavonoid pathway, present in several plant species and tissues.
The interest in such compounds originates for their multiple utilisation, in industrial activity, but mainly for the beneficial effect of their presence in food and feed.
Since their share the biosynthetic pathway with anthocyanins it has been possible to identify and clone structural and regulatory genes in several species and describe the regulatory mechanism controlling their biosynthesis: three actors WD40-bHLH and MYB interact to activate/repress this route. Among this ternary complex, the MYB partner seems to be main player in specifying the competence of a given organ/tissue to accumulate CT. A large amount of MYB genes have been isolated from several species. They can exert both positive and negative action on the pathway.
In this work we tested the effects of the expression of *VvMYBPA1* in different tobacco tissues to test the possibility to use this gene as an activator of this route in different crop species.
In grape, *VvMYBPA1* is expressed in flowers and at the early stages of berry development, its expression correlates with CT accumulation and this gene complements *TT2* in *tt2* Arabidopsis mutants (Bogs et al, 2007).
Here we report on molecular and metabolic characterization of 3 T_2 progeny of tobacco plants transformed with *VvMYBPA1*. These plants have been selected for their different floral phenotypes: ranging from whitish to pale pink limbs, whereas the control plants show pink floral limb. Notably, the reduced accumulation of anthocyanins correlated inversely with the accumulation of CT and positively with the quantitative expression of the transgene. Among the genes tested CHS and ANR resulted the most positively affected by *VvMYBPA1.*

Metabolic analysis of flower tubes and limbs have shown that beside the anthocyanin derivatives, 41 additional phenolic compounds show different concentrations in the transgenics. Among those metabolites whose levels were markedly different between transgenic and control lines are the flavan-3-ols, catechin and epicatechin, the building blocks of CT, and their dimers and polymers. Notably, the concentration of epicatechin, that is about an order of magnitude higher than catechin in any genotype investigated, increased almost linearly with the decrease of the anthocyanin content. Interestingly, none of these compounds have been detected in wild type tubes. Other compounds such as chlorogenic, cryptogenic and neochlorogenic acid followed the same rule as the flavan-3-ols. In particular, chlorogenic and neochlorogenic acids displayed an increment in their accumulation both in limbs and tubes of the transgenic lines; interestingly, this increment mirrors the steady state levels of the transgene to suggest a specific action of the exogenous transcription factor on this branch of the phenylpropanoid pathway.

P-23

Abiotic stress modulates biosynthesis of polyisoprenoid alcohols in *Arabidopsis thaliana* hairy root culture

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Polyisoprenoid lipids are representatives of specialized metabolites widespread in all eukaryotic cells. Polyisoprenoids, i.e. dolichols and polyprenols accumulated in plants are derived from both isoprenoid-generating pathways – the mevalonate (MVA) and methylerythritol phosphate pathways (MEP). Dolichols are involved is numerous vital cellular processes, e.g. protein glycosylation and plant adaptation to adverse environmental conditions.

In this study the effect of osmotic stress and heavy metal salts on polyisoprenoid alcohol biosynthesis in Arabidopsis hairy roots was analyzed. Supplementation of the culture medium with sorbitol or cadmium chloride resulted in an increased dolichol accumulation (approx. 160% and 150% of the control, respectively). Simultaneously, sorbitol modulated the expression of *cis*-prenyltransferases (CPTs, enzymes responsible for formation of the dolichol carbon skeleton) by inducing *AtCPT3, AtCPT6* and *AtCPT7* (up to 6-fold increase for *AtCPT6*) and diminishing *AtCPT1* and *AtCPT2* transcript level. Cadmium chloride slightly induced the expression of *AtCPT3* and *AtCPT7* (180% of control) and simultaneously inhibited the expression of all the remaining AtCPT encoding genes.

Metabolic labeling with a general precursor ([^{13}C]glucose) and pathway-specific precursors ([^{2}H]mevalonate and [^{2}H]deoxyxylulose) was applied to elucidate the involvement of the MVA and MEP pathway to dolichol biosynthesis upon osmotic stress (0.3M sorbitol). Preliminary results indicate that osmotic stress, at least at the applied experimental conditions, does not alter the contribution rate of the pathways to the dolichol accumulation.

Results of these experiments indicate that enhanced dolichol biosynthesis is an integral part of the cellular metabolic pathways

reprogrammed in plants cell in response to the abiotic stress and both pathways, the MVA and MEP, are induced to produce required polyisoprenoids.

P-24

The proteome of a toxic vacuole: a tale of struggle and survival in the leaves of the alkaloid producing plant *Catharanthus roseus*

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Vacuoles play an array of key roles in growth, development and environmental interactions in plants, including the accumulation of specialized metabolites that can thus exert their defence toxic effect without interfering with cell physiology. However, many aspects of vacuole multifunctionality are still poorly understood. Here, the leaf vacuolar proteome of the medicinal plant *Catharanthus roseus* was thoroughly characterized, aiming to obtain the functional portrait of this alkaloid accumulating, toxic organelle, and to identify candidate genes implicated in alkaloid metabolic fluxes. Whole vacuoles and tonoplast vesicles were isolated with high purity and the obtained peptide sequence information allowed the identification of 1872 putative proteins. Results indicated a high commitment of vacuoles with defence specialized metabolism, redox homeostasis and stress response, apart from the well-known involvement in lytic reactions. *C. roseus* vacuoles also present signs of a very significant primary metabolism and signalling activity. Cytochrome P450 electron transfer chains and a very high number of ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) transporters were unequivocally identified, including several candidates to alkaloid metabolic fluxes. Overall, the detailed analysis of the identified complement of vacuolar proteins sets forth important new conclusions concerning the multiple roles of vacuoles.

P-25

Housekeeping gene selection for expression level normalization in the medicinal plant *Catharanthus roseus*

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The leaves of *Catharanthus roseus* accumulate in very low levels the terpenoid indole alkaloids (TIAs) vinblastine and vincristine, which are widely used in anticancer treatments, making of this plant an object of intensive study. Recently, transcriptomic studies have enabled the identification of many candidate genes potentially involved in TIA metabolism. One of the first tools used for functional characterization of such candidates is gene expression analysis by Quantitative Real-Time PCR (qPCR), which requires normalization using reference genes showing stable expression throughout a number of organ, tissues and cell types involved in TIA metabolism. Here, a previously performed differential cDNA-AFLP transcriptomic profiling of several *C. roseus* organs and cell-types was explored for the identification of reference gene candidates showing expression stability, namely in leaf idioblast cells, which are specifically involved in the late, rate-limiting steps of TIA biosynthesis. *In silico* analysis of 21 candidate tags using available *C. roseus* transcriptomic databases enabled the selection of three genes for evaluation: 40S ribosomal protein S28 (*RPS28*), 60S ribosomal protein L24 (*RPSL24*) and ADP-ribosylation factor 2 (*ADP2*).

qPCR analysis using different *C. roseus* organs, suspension cells and idioblasts revealed that these three genes have a high expression stability and are more suitable to be used as reference genes than 40S Ribosomal protein S9 (*RPS*9), commonly used for normalization of expression studies in *C. roseus*.

P-26

Enhancing levels of aromatic amino acids in plants leads to improved fragrance in flowering petunia and to enhanced health-related metabolites in grape derived cell culture

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Aromatic Amino Acid (AAAs) derived specialized metabolites are important compounds synthesized widely by many agricultural crops. They are involved in the adaptations of plants to biotic and abiotic stresses, contributing to plant's defence mechanisms as well as to their performance. AAA derived compounds are commercially significant as pigments, aroma compounds and phyto-antioxidants. Despite their significance, attempts to improve these traits by classical breeding, without affecting plant's vigour, gained little success.

Here, we studied the profile of secondary metabolites of a purple flowered petunia cultivar and a red grape cell culture, expressing a feedback-insensitive bacterial form of 3-deoxy-diarabino-heptulosonate 7-phosphate synthase enzyme (AroG*) of the shikimate pathway, as a tool to stimulate the conversion of primary to secondary metabolism via the aromatic amino acids. In both plant systems, the presence of AroG* resulted in a significant increase in AAA levels, and enhanced levels of the downstream specialized metabolites, without affecting plant morphology.

In AroG* petunia, the petals accumulated significantly higher levels of fragrant benzenoid-phenylpropanoid volatiles, without affecting the flowers' lifetime. In contrast, AroG* abundance had no effect on flavonoids and anthocyanins levels. In the grape cell culture, AroG* led to accumulation of resveratrol and quercetin, known for their health

promoting characteristics. Here too increased AAA production did not lead to increased anthocyanin pigmentation, suggesting that the control mechanism of the anthocyanin pathway in grape fruits and in petals of flowers is independent of AAA concentrations.

Selected References:

Oliva et al., 2014 (Plant Biotechnology Journal): Enhanced formation of aromatic amino acids increases fragrance without affecting flower longevity or pigmentation in Petunia × hybrid

Manela*, N., Oliva, M*., et al., In Preparation. (*equal contribution).): Phenylalanine and tyrosine levels are rate limiting factors in production of health promoting metabolites in grape cell suspension

P-27

Towards metabolic engineering of lupeol in pepper: cloning and characterization of lupeol synthase

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Pepper fruits are an excellent source of health-related compounds, such as vitamins (ascorbic acid), carotenoids (provitamin A), tocopherols (vitamin E), flavonoids, capsaicinoids and triterpenes including lupeol. The latter compound shows interesting pharmacological activities including anti-inflammatory, anti-tumor, anti-mutagenic and anti-malaria properties. Moreover, it has been demonstrated that dietary supplementation of lupeol prevents cancer and coronary diseases.
Lupeol biosynthesis occurs in the citosol through the mevalonate (MVA) pathway that leads to the formation of 2,3-oxidosqualene, which is then cyclized in lupeol by lupeol synthase.
In this work, using the *lupeol synthase* gene isolated from *Solanum lycopersicum* as the query in a BLAST analysis identified the full-length cDNA of a *lupeol synthase* from pepper (*Capsicum annuum* L.), designated *CaLUP*. The *CaLUP* open reading frame, consisting of 2271 bp, was predicted to encode a protein of 756 amino acid residues. The comparative and phylogenetic analyses of CaLUP showed that this protein is closely related to other plant lupeol synthases, sharing 66–90% identity with their amino acid sequences. In particular, a high degree of identity (80%) was observed with lupeol synthase of *Solanum tuberosum*.
Alignment between the genomic and cDNA sequences revealed that the *CaLUP* gene was organized into 18 exons and 17 introns. Intron lengths ranged between 83 bp (intron 5) and 7845 bp (intron 7), and altogether they covered 92% of the *CaLUP* genomic region. Genotype- and tissue-specific expression patterns of *CaLUP* were evaluated by quantitative real-time PCR analysis (qRT-PCR).
In order to understand the transcriptional regulation mechanisms, the upstream region of *CaLUP* was isolated and by scanning the PLACE database, several *cis*-regulatory elements were identified as hormone responsive, stress responsive and light responsive. Based on these results, we selected four elicitors for *in vitro* and *in-vivo* treatment of

plantlets, and the expression levels of *CaLUP* were determined by qRT-PCR.
Since cell cultures represent an ideal system for the elicitation treatments, we have established cell suspension cultures of *C. annuum* cv. Quadrato d'Asti using friable calli**.**
For functional validation, *CaLUP* expression in yeast.was investigated For this purpose, *CaLUP* was cloned into pYES2 expression vector under GAL promoter and it was then transformed into *S. cerevisiae* strain INVSc1.
Cloning and characterization of *CaLUP,* a key gene of the lupeol pathway, pave the way for metabolic engineering approaches of this valuable compound in pepper.

P-28

Metabolic Engineering of carotenoids in potato affects ABA metabolism and tuber shelf-life

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Carotenoids are secondary metabolites involved, in animals, in the prevention of several animal diseases including cancers and cardio-vascular pathologies. In plants, they play essential functions as photosynthetic pigments in leaves, secondary metabolites in fruits and flowers, and hormone precursors (ABA and strigolactones); three out of them (α-/β-carotene, β-cryptoxanthin) cover a fundamental role in human nutrition as precursors of vitamin A.

We have previously generated potato transgenics enriched in β-carotene and total carotenoids, so defined “Golden”, by expressing simultaneously three genes of bacterial origin (*CrtB*, phytoene synthase; *CrtI*, phytoene desaturase; *CrtY*, lycopene β-cyclase). In a different approach, potato tubers have been engineered with the “Or” gene from cauliflower, resulting in β-carotene and total carotenoid levels.

We have performed a global profiling at transcriptional (RNA-Seq), metabolomic (LC-MS) and phenomic level on all these materials. Integration of Gene expression profiles, integrated and high-throughput metabolomics, revealed unscheduled transcript-metabolite correlations, both in primary (aa, lipids etc) and secondary (alkaloids, phenylpropanoids) metabolism, shedding light on novel co-regulatory dynamics which emerged in tuber metabolism and, limited to “Golden” samples, in post-harvest storage. “Golden” tubers also displayed higher levels in ABA and a significant reduction in starch content.

A strong influence of carotenoid/ABA accumulation on tuber maturation kinetics was observed, while GO enrichment analysis allowed identification of gene classes specifically regulated in "Golden"/"Or" tubers. Overall, these analyses revealed the central role of b-carotene/ABA in regulating tuber metabolism and development and large-scale network analysis proved to be a valuable approach for rational design of new biofortified crops by through identification of higher correlative power nodes (hubs of the network), as potential targets in future breeding programs.

P-29

A novel R2R3 AaMYB1 promotes artemisinin and gibberellins biosynthesis as well as trichome development

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The effective anti-malarial drug artemisinin isolated from *Artemisia annua* is relatively expensive because of the low content in the plant as it is only synthesized inside the trichomes. Therefore, genetic engineering of *A. annua* is one of the most promising approaches to improve the yield of artemisinin. In this work, a R2R3 MYB transcription factor (*AaMYB1)* has been identified, and when overexpressed in *A. annua* under the control of a trichome specific promoter (*pCYP71AV1::AaMYB)* essential artemisinin biosynthetic genes are highly upregulated. As expected, phenotypical analyses show that the amount of artemisinin was also increased,up to 2-fold, in these transgenic plants. In order to study the possible effects of AaMYB1 on other biological processes as trichome development and gibberellin (GA) biosynthesis, AaMYB1 was also constitutively overexpressed in *Arabidopsis thaliana* using the CaMV35S promoter. Here, we show that AaMYB1 affects both trichome initiation and branching in *Arabidopsis*. Molecular analyses conducted show that two crucial trichome activator genes are both up-regulated in plants overexpressing the *AaMYB1* gene. Moreover, AaMYB1 also controls GA biosynthesis in *Arabidopsis* by positively affecting the expression of the enzymes that convert GA_9 into the bioactive GA_4 as well as the enzymes involved in the degradation of GA_4 into inactive GA_{34}. Finally, the Arabidopsis *AaMYB1*ortologue was identified, *AtMYB61* of. AtMYB61 is also able to transcriptionally activate, at least partially, essential genes of the trichome initiation and branching pathways as well as up-regulate other crucial genes of GA biosynthesis and degradation.

P-30

Conversion of the monoterpenoid indole alkaloid vinorine by recombinant P450 3A4

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Since the isolation of ajmaline from root tissue of *Rauwolfia serpentina* (L.) Benth. ex Kurz in 1931 (Siddiqui & Siddiqui, 1931), there has been an increasing interest in decoding the biosynthetic pathway of this important representative of the monoterpene indole alkaloid family.
Initially, the catalytic activities of all involved enzymes could be detected in the crude plant extract and the majority were identified and isolated. To accomplish the decisive step towards the understanding of the whole pathway, current research has focused on the remaining five (the sarpagan bridge enzyme, vinorine hydroxylase, vomilenine reductase, dihydrovomilenine reductase and acetylnorajmalan methyltransferase).
Within this context, major efforts in the characterization of the vinorine hydroxylating enzyme (VH) were achieved in 1995 (Falkenhagen & Stöckigt, 1995). Several *in vitro* tests with microsome preparations of *R. serpentina*, purified vinorine and NADPH led to the prediction of cytochrome P450 involvement. Unfortunately, to date, this specific enzyme has not been identified amongst the variety of cytochrome P450 isoforms characteristic of the *R. serpentina* genome. As an alternative approach, already available P450 candidates were verified for the desired enzymatic activity. In particular, cytochrome P450 3A4, as a catalyst of a multitude of substrates of various sizes and characteristics, was tested *in silico* for binding with vinorine. The final result was the theoretical confirmation of compatibility of vinorine with the substrate binding site of cytochrome P450 3A4.
In the performed follow-up studies, an appropriate *in vitro* assay was established, confirming enzymatic conversion of vinorine by cytochrome P450 3A4 resulting in a product of molecular mass corresponding to that of vomilenine. However, as indicated by HPLC analysis, the generated compound exhibited different polarity relative to the anticipated VH product. Whether the reaction led to an epimeric or a positional isomeric structure of vomilenine has yet to be determined.

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Siddiqui, S., Siddiqui, R. H. (1931). "Chemical examination of the roots of Rauwolfia serpintina." Journal of the Indian Chemical Society, 8, 667-680

P-31

Metabolic engineering of secondary biosynthetic pathways in plants

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Modification of known, pharmacologically important plant natural products, like monoterpenoid indole alkaloids, by halogenation can introduce novel biological functions into given metabolites *in planta*. In addition, further modifications, like substitution of the indole ring system, are made easier by previous chlorination or bromination. Such metabolic engineering can be performed by halogenation of precursor molecules, like tryptophan, through introduction of flavin-dependent tryptophan halogenases. These enzymes, originating from bacteria, led to generation of halogenated indole alkaloids in *Catharanthus roseus* (Runguphan *et al.* 2010). To further verify this principle by means of transposition of the ajmaline biosynthetic pathway into an alternative plant, all relevant precursors, such as secologanin, tryptamine and strictosidine, needed to be synthesized *in planta*. Therefore, eleven genes of the strictosidine biosynthetic pathway as well as one halogenase (rebHY455W) and one reductase gene (rebF) were introduced to the genome of tobacco for transient and stable expression. Initial LC-MS analyses proved the biosynthesis of eleven novel compounds after transient transformation of *Nicotiana benthamiana*.

In a second approach, substrate specificity of tryptophan halogenases was tested on indole derivatives in the indoxyl biosynthetic pathway (Warzecha *et al*. 2007). Formation of the blue pigment indigo, naturally found in dye plants like *Indigofera tinctoria*, results from dimerization of two indoxyl molecules in the presence of oxygen. To analyze the ability of three different halogenases, RebHwt, RebHY455W and Stth, to chlorinate indole and indoxyl, the relevant genes were co-expressed with *bx1* and *2A6*, encoding for an indole synthase from maize and a human cytochrome P450, respectively.

Runguphan, W., X. Qu and S. E. O'Connor (2010). "Integrating carbon-halogen bond formation into medicinal plant metabolism." Nature 468(7322): 461-464.

Warzecha, H., A. Frank, M. Peer, E. M. Gillam, F. P. Guengerich and M. Unger (2007). "Formation of the indigo precursor indican in genetically engineered tobacco plants and cell cultures." Plant Biotechnol J 5(1): 185-191.

Session III

Industrial applications of plant secondary metabolites

O-17

Key-note lecture

Production of immunoglobulins in plants-challenges and solutions

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O-18

Ketocarotenoid-rich tomato powders incorporated into natural-matrix microspheres: stability and controlled release for fish feeding and biomedical applications

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Recent advances in metabolic engineering recently applied for tomatoes, to be enriched in ketocarotenoids (mainly astaxanthin) were recently reported (EU-FP7 project METAPRO) and opened new opportunities to replace the synthetic pigments by natural pigment-rich products to be used as food and feed ingredients or additives with enhanced nutritional or commercial value.
Three high astaxanthin (HA), high ketocarotenoid (HK), high lycopene(HL) tomatoes obtained by metabolic engineering were characterized comparatively with control genotypes (C). Shortly, freeze dried samples of the four above-mentioned tomato types were analyzed for their content in specific carotenoids, fingerprinted by UPLC-QTOF-MS, identifying and quantifying the individual molecules (carotenoids and polyphenols). By an original microencapsulation technology, microspheres (of 1-1.5 mm diameter) containing 5% tomato (HA, HK, HL and C) powders were incorporated into natural matrices (alginates and coated with chitosan), in order to improve the pigment stability and bioavailability. All microspheres were characterized for their morphology and tested for pigment stability during 12 months, as fresh or freeze dried, stored in 3% saline water, as well checked for controlled release in a simulated gastric and/or intestinal environment. To check the comparative composition of the four sample types, as such or after encapsulation and storage, different spectrometry methods (UV-Vis, FTIR, direct injection - MS) and UPLC-QTOF-MD analyses were applied. High keto-carotenoid microspheres proved good stability in saline water for 12 months and are highly

recommended as feed supplements for fish feeding, while high astaxanthin microspheres are recommended as food supplements which allow controlled release of the pigment and assures protective, antioxidant activity in gastric and intestinal environment.

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O-19

Betulins as novel lead compounds for cancer

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Betulin and resin acids are common by-products of the forest industry. The outer bark of white birches contains up to 35% dry weight betulin, and currently represents a low-value waste product with no economically significant use. Betulin is an extremely hydrophobic pentacyclic triterpene alcohol which serves an interesting starting point for chemical and biochemical synthesis of its derivatives. Plant cells exhibit a large biochemical potential for production of compounds, many with highly complex structures. Through biotransformation, catalyzed by cells, the diversity of potential bioactive derivatives can be increased.

Our objective was to synthesize novel fine and specialty chemicals from betulin, and to characterize their properties by both medicinal chemistry and *in vitro* biotransformation in terms of biological and pharmacological activity. We have made more than 100 synthetic betulin-derived compounds which have been tested in a systematic way using advanced *in vivo* cell-based functional primary and secondary screens. Organotypic model system was utilized to address the proliferation and invasiveness of an aggressive prostate cancer cell line. Compounds were screened in both 2D and 3D growth conditions, and the most promising 25 leads were selected for sensitivity tests using a panel of other cell lines. Six compounds were of special interest as they showed specific anti-invasive properties in mechanisms of action and structure –activity-relationship studies. We have also established experimental biotransformation conditions for these poorly soluble betulin derivatives using living plant cells. The cell suspension cultures of tobacco SR1 line were fed with the synthetic betulin-derived alkaloid and its conversion to new derivatives was investigated. This presentation highlights the characterization and bioactive properties of betulin-derived compounds obtained through chemical synthesis and *in vitro* biotransformation.

O-20

Diadenosine triphosphate is a novel factor which synergistically with cyclodextrins enhances the biosynthesis of *trans*-resveratrol in suspension cultured cells of *Vitis vinifera* cv Monastrell

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Dinucleoside polyphosphates like diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A) are considered as signal molecules because they may trigger defence responses when plant cells are under stress. Indeed, Pietrowska-Borek et al., (2011) demonstrated that the addition of micromolar concentrations of Ap_3A or Ap_4A, to *Arabidopsis thaliana* seedlings provoked a remarkable increase of gene expression especially those encoding enzymes involved in the phenylpropanoid pathway (phenylalanine ammonia lyase (PAL) and 4-coumarate CoA ligase (4CL) which leads to the formation of flavonoids, lignins and stilbenes. Other molecules involved in the induction of secondary metabolite pathways, and which take part in plant cell defence reactions are cyclodextrins (CDs), cyclic oligosaccharides consisting of seven glucopyranose residues linked by α (1→4) glucosidic bonds. In fact, the biosynthesis of *trans*-resveratrol, one of the basic units of stilbenes which derives from the phenylpropanoid pathway, was stimulated by adding CDs to suspension cultured cells of *Vitis vinifera* cv Monastrell (Belchí-Navarro et al., 2012). This eliciting activity of CDs was due to their chemical similarity to the alkyl-derived oligosaccharides that are released from plant cell walls during a fungal attack (Bru et al., 2006). In this work, we show that the addition of Ap_3A in combination with CDs to *V. vinifera* suspension cultured cells enhanced synergistically the biosynthesis of *trans*-resveratrol, which is secreted and accumulated, in a great extent, into the spent medium. Although these elicitors specifically induced the expression of phenylpropanoid pathway genes *PAL*, cinnamate-4-hydroxylase (*C4H*)

and *4CL* and the stilbene synthase1 gene responsible for the formation of *trans*-resveratrol, no synergistic effect was observed in the gene expression when the combination of Ap_3A and CDs was used.

References:

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Belchí-Navarro et al., 2012. Enhanced extracellular production of *trans*-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate, Plant Cell Rep. 31:81–89.

Bru et al., 2006. Modified cyclodextrins are chemically defined glucan inducers of defense responses in grapevine cell cultures, J Agric Food Chem.54:65-71.

O-21

Effect of the mycotoxin ophiobolin A on cell proliferation, cell viability and redox state on tobacco Bright Yellow-2 cells

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Ophiobolin A is a sesterpenoid produced by phytopathogenic fungi, which attack several plants of agronomic interest. These compounds have been reported to have specific toxic effects on different cancer cell lines, but their mode of action is still unclear. Ophiobolin A induced different outcomes in plant tissues depending on its concentration. When tobacco Bright Yellow-2 (BY-2) cells were treated with 10µM ophiobolin A, programmed cell death (PCD) was triggered. Unlike most plant PCDs previously reported, ophiobolin A caused a PCD that was not mediated by an early overproduction of reactive oxygen species and did not involve alterations in the ascorbate-glutathione (ASC-GSH) metabolism, at least during the first hours after PCD induction. On the other hand, lower concentrations of ophiobolin A did not affect cell viability but arrested cell cycle in S/G2 in a reversible manner. Concomitantly, it froze the activity of the poly ADP-ribose polymerases (PARPs), nuclear enzymes involved in DNA metabolic transitions, which normally increases during the exponential growth phase in plant cells. Moreover, several parameters correlated to glutathione metabolism resulted to be impaired by ophiobolin A, such as the variations in glutathione levels that characterize the growth curve of control cells, glutathione redistribution between nucleus and cytoplasm and protein glutathionylation profiles. The obtained data sheds new light on the relevance of redox control and redox changes occurring at specific cellular compartments, as pivotal factors driving cell proliferation.

O-22

Plant tissue cultures as tools for the production of bioactive ingredients with cosmetic applications.

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Plants remain a major source of extracts or purified compounds for cosmetic use. Cosmetic research is constantly looking for innovative plant ingredients, which must be guaranteed for quality and safety to the final consumers. Unfortunately, many plant derived products can be used limitedly because several plants of cosmetic interest may contain toxic compounds, can be subjected to diseases and differ in the content of metabolites seasonally and from harvest to harvest. By using biotechnological approaches, plant cells and tissues can be easily cultivated in sterile conditions, totally independently of geographical and climatic factors, thus represent a very relevant system for the production of valuable metabolites. Moreover, differently from plants grown in the field, plant culturing perfectly responds to the today's market demands of safety, quality and sustainability.

By exploiting the natural biodiversity and versatility of the plant cells, suspension cultures have been successfully used as multiple sources of cosmetic active ingredients having different chemical nature, and a wide range of cosmetic activities. Besides plant cell cultures, alternative biotechnological approaches can be adopted in case active ingredients with more restricted and specific functions were required. One example is provided by the generation of hairy root cultures in the laboratory, which represent a novel and promising system for the production of certain class of more specific secondary metabolites with interesting cosmetic applications.

Session III Posters

Industrial applications of plant secondary metabolites

P-32

Towards a platform organism for terpenoid production – *in silico* comparison of *E. coli* and *S. cerevisiae* as potential hosts

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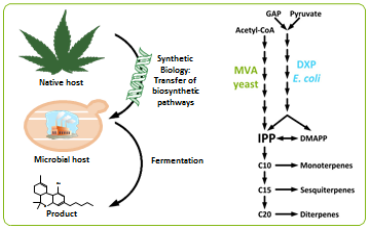
Terpenoids are one of the largest classes of natural products and they possess important medicinal and industrial properties. The heterologous production of plant terpenoids in microorganisms is a concept to overcome supply problems and high purification costs as several compounds are rare and produced only in low amounts in plants [1]. Our focus is on the development of a platform organism for the efficient supply of isopentenylpyrophosphate (IPP), the biosynthetic precursor of all terpenoids. *E. coli* and *S. cerevisiae* are potential hosts that use two different pathways to produce IPP. In this study *E. coli* and *S. cerevisiae* are compared *in silico* by means of elementary flux mode analysis (EMA) regarding their metabolic potential to supply IPP. EMA allows the calculation of a solution space containing all steady state flux distributions of a metabolic network considering stoichiometry, topology and thermodynamics [2]. The theoretical maximum IPP yield is calculated, which can be used for the estimation of the potential efficiency of a process. Exchange and combination of the DXP and MVA pathway as well as optimal flux distributions are analyzed providing a basis for rational strain design.



Data published in:
Gruchattka, E., Hädicke, O., Klamt, S., Schütz, V., Kayser, O. (2013) In silico profiling of *Escherichia coli* and *Saccharomyces cerevisiae* as terpenoid factories. Microbial Cell Factories, 12: 84

P-33

Biotechnological taxol production and excretion in *T. media* cell cultures under elicitation. A transcriptomic and metabolic study.

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Taxol is a complex diterpene alkaloid with an intense antitumor activity, widely used for the treatment of several types of cancer. At present, for a variety of reasons, the growing demand for taxol greatly exceeds the supply that can be sustained by isolation from its natural source, the inner bark of several *Taxus* species, and the most promising approach for a sustainable production of taxol and related taxanes at an industrial level is provided by plant cell cultures. Culture supplementation with elicitors has proven to be the best strategy for increasing the production of this anticancer agent. In this study, the effect of cyclodextrins (CDs), with or without methyl jasmonate (MeJA), on taxane production and the expression of several genes involved in taxane biosynthesis has been explored in a two-stage cell culture of *Taxus media.* The biosynthetic pathway leading to taxol is highly complex, with several steps still undefined, so to obtain new insights into this process and its control, it is essential to know how the different factors improving taxol production affect the gene expression and metabolic profiles in *Taxus* cell cultures. Our results have shown that joint elicitation with MeJ and CDs induces an important reprogramming of gene expression in *T. x media* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes observed in this study.

It should be emphasized that although the expression levels of all studied genes increased significantly in the presence of both elicitors, those encoding the transferases DBAT, BAPT and DBTNBT showed much lower transcript accumulation than the genes encoding TXS, PAM and several hydroxylases. This indicates that the three

transferases active in the final part of the taxane biosynthetic pathway may control flux-limiting steps leading to taxol.

It should also be emphasized that the presence of both cyclodextrins and MeJA in the culture not only enhanced the production of taxanes (over 31-fold increase in taxol production compared with control cultures), but also their excretion to the culture medium (more that 90% of the taxol produced in the cells). This steady rate of taxol release could explain the active biosynthesis and extracellular accumulation observed, since it would clearly diminish the usual feedback inhibition processes and/or toxicity resulting from the presence of taxol in the cytoplasm.

P-34

Biotechnological production of piceatannol in transgenic tobacco plants and cell suspensions.

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Piceatannol (3,5,3´,4´-tetrahydroxystilbene) (Pt) is a hydroxylated natural analogue of resveratrol (R), a natural product well-known for its anti-ageing, anti-inflammatory, and cancer chemopreventive effects. Recent studies attribute Pt with greater bioactivity than R. It has been described that R acts as a pro-drug, being converted into Pt in the human liver by CYP1B1, a P450-dependent hydroxylase. In contrast to R, there is not any known abundant natural source of Pt suitable for use as a production platform. In this work, we have studied the potential of producing Pt in plants through the expression of *HsCYP1B1* gene in two tobacco systems. First, *Nicotiana benthamiana* agroinfiltration with two *Agrobacterium* strains bearing a grapevine stilbene synthase and human liver CYP1B1, respectively, resulted in the accumulation of three stilbenoid products: R, Pt and piceid (a glycosylated R derivative). Second, tobacco hairy root lines carrying the *HsCYPB1* gene were obtained after the infection of *N. tabacum* leaves with *A. rhizogenes* A4 containing the plasmid pK7WG2 carrying the indicated gene. Cell lines were obtained by dedifferentiation of the hairy root lines by treatment with IAA and kinetin. Cell suspensions were fed with R, with or without cyclodextrins. Our results show the ability of the tobacco cell line to accumulate R intracellularly and to convert it into Pt. The cyclodextrin treatment improved the R biotransformation leading to the extracellular accumulation of Pt.

The results provide evidence that in tobacco leaves and cell cultures, CYP1B1-mediated synthesis of Pt represents a promising platform for Pt bioproduction.

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Analysis of proanthocyanidins in bark from Norwegian trees

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Bark - a sawmill by-product - can be an important raw material for the production of biopesticides or feed additives. Bark contain proanthocyanidins (PAs) - condensed tannins - that are flavanoid polymers believed to play an important role in a tree's defense towards external invasions by wood-rotting fungi or insects. Proanthocyanidins have also been shown to prevent infections of parasitic worms and to have nutritional benefits when ingested by animals. Before any utilization of bark, a quantification of the amount of proanthocyanidins in the bark material is needed. We have analyzed both free and bound proanthocyanidins in bark from cut trees of Scots pine (*Pinus sylvestris* L.), Norwegian spruce (*Picea abies* L.) and downy birch (*Betula pubescens*) sampled in the municipality of Tingvoll, Norway, in April 2013. Free PAs in bark were extracted with 70% aqueous acetone and determined by a gravimetric Yb(III) precipitation method (Giner-Chavez *et al.* 1997) at the Norwegian University of Science and Technology. Total PAs in bark (sum of bound and free PAs) were determined by HPLC-PDA after depolymerization of the PAs in the presence of a nucleophile (Hellström *et al.* 2008) at MTT Agrifood Research Finland. Although a limited number of samples were analyzed, the study indicated that only a small part of the bark PA was free and readily available for extraction, while the majority was strongly bound to the fiber and could only be liberated by chemical degradation. The PA content was highest in bark from the younger trees (≤ 40 years old; max. 8.5% PA content). The study has given insight into proanthocyanidin levels in Norwegian bark and an important step toward large scale production of PAs from barks and their utilization as biopesticides and feed additives.

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Session IV

Future aspects and perspectives

O-22

Sören Bak, DK
TRIFORK (to be confirmed)

O-23

From *DISCO*very to products: A next generation pipeline for the sustainable generation of high-value plant products

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DISCO is an academic/industry alliance, consisting of pan-European and IPCP partners with complementary multidisciplinary expertise. The overall aim and concept of DISCO is a generic pipeline from discovery to industrial valorisation, using the very latest enabling technologies, to deliver sustainable biosources of plant derived products. A key feature of the DISCO project is its potential to utilise and build on existing and previous EU investments, rapidly and efficiency transferring the tools and strategies developed to new plant derived target molecules. The bioactives and high-value compounds targeted in DISCO are carotenoids, other terpenoids and tropane alkaloids. These targets all desperately require the development of new sustainable biosources and "greener" production chemistries.
The research and demonstration activities of DISCO are industry driven and will:

- Exploit existing and evolving biodiversity in Solanaceae and Iridaceae to perform bioprospecting with state of the art metabolomic approaches for the targeted molecules of interest.
- Use transcriptomics and network biology approaches to elucidate new biosynthetic and regulatory pathway components and their alleles involved in the formation of the targeted bioactives/high-value phytochemicals.
- Develop and incorporate enabling technologies into discovery, application and translational pipelines.
- Generate new biosources of high value carotenoids, terpenoids and tropane alkaloids by metabolic engineering and molecular breeding approaches.
- Develop down-stream processes and integrative biorefining strategies for co-product and biomass utilisation that reduce environmental impact.

- Demonstrate production feasibility and product effectiveness beyond the present state of the art.

O-24

Transgenic vs. non-transgenic metabolic engineering of carotenoids in tomato

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Metabolic engineering to enhance the production of high value plant natural substances is frequently considered from the perspective of transgenic interference (DNA-mediated genetic modification, GM). Nevertheless, in certain cases non-transgenic modifications can potentially achieve similar results. In view of the current attitude toward food products made from GM plants, especially in the health food niche, a non-GM metabolic engineering should be considered. An assessment of GM vs. non-GM approaches to optimize the production of useful compounds in plants will be described in the case of carotenoid. Tomatoes, fresh or processed, constitute a major dietary source of carotenoids. While specific health benefits of tomato consumption have been attributed to lycopene and apocarotenoids derived from it, recent studies have suggested that phytoene and phytofluene (P&P), which occur in human plasma and tissues, exhibit significant anti-oxidative capacities in humans (Reviewed by: (Engelmann *et al.*, 2011; Melendez-Martinez *et al.*, 2015). P&P are colourless intermediates in the carotenoid biosynthesis pathway and are present in plant tissues at low levels. Metabolic engineering of carotenoids by means of GM has been successfully implemented in tomato fruits to elevate the level of trans-lycopene or to produce different carotenoids. Using a non-GM strategy we have developed tomato varieties that accumulate high concentration of P&P in fruits. This achievement will be discussed in comparison to other GM manipulations of carotenoids in tomato.

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DISCOvery to products: A next generation pipeline for the sustainable generation of high-value plant products.

O-25

Saffronomics: Transcriptomics-based dissection of the saffron stigma apocarotenoid pathway

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Crocus sativus stigmas are the source of the most expensive spice on Earth, saffron, and owe their characteristic color, taste and aroma to the apocarotenoids crocetin, crocins, picrocrocin and safranal. The identification of genes catalyzing apocarotenoid biosynthesis and sequestration is a necessary prerequisite for the engineering of the pathway in heterologous systems. Through deep transcriptome sequencing at various stages of stigma development, we identified candidate genes for the whole biosynthetic pathway, from the initial cleavage of zeaxanthin, to the cytoplasmic glycosylation of crocetin, to the vacuolar sequestration of glycosylated crocins.
CCD2, a member of a novel CCD clade, mediates the two-step conversion of zeaxanthin to crocetin dialdehyde via 3-OH-β-apo-8'-carotenal. Similar to CCD1, CCD2 has a cytoplasmic localization, suggesting that it may cleave carotenoids localized in the chromoplast outer envelope (1). The immediately downstream step, dehydrogenation of crocetin dialdehyde, is also being investigated *in bacterio* by coexpression of different stigma dehydrogenases with CCD2. At the other end of the pathway, we identified ABC and MATE-type transporters expressed at different stages of stigma development, when apocarotenoid and flavonoid compounds are accumulated. Heterologous expression in yeast, coupled with in vitro transport assays, is being used to dissect the substrate specificities of the different transporters.

The research has benefited of the networking activities of the COST Action FA1101 “Saffronomics” and in particular of WP1 of the action, aimed at developing genomic tools in Crocus sativus.

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O-26

A library of TALE-activated synthetic promoters: application for metabolic engineering in plants.

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Engineering of complex metabolic pathways in stable transgenic plants requires the coordinate expression of pathway genes in targeted tissues. One strong limitation is the number of available promoters that are expressed in a specific tissue and exhibiting different levels of expression. As a solution to this problem we have developed a library of 48 synthetic promoters that are controlled by a single designer Transcription Activator-Like Effector (TALE). In transient assays in *Nicotiana benthamiana* the expression strength provided by these promoters ranged from a few percent to almost 100% of that of the 35S promoter. The utility of these promoters for metabolic engineering was first confirmed in transient assays by expressing genes for the biosynthesis of plant diterpenoids. We next generated transgenic tobacco and Arabidopsis plants expressing diterpenoid biosynthesis pathways in different tissues, such as glandular trichomes and the epidermis. Progress on these experiments and the potential of these synthetic promoters for metabolic engineering and regulatory circuit engineering in plants will be presented.

O-27

Plant made recombinant pharmaceuticals - where is the field?

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O-28

Refinement of the standards for Genetic Design in Plant Synthetic Biology using the GoldenBraid format

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Synthetic Biology-inspired Modular DNA assembly methods are being increasingly employed in Plant Metabolic Engineering projects. Modular cloning facilitates genetic engineering by providing simple rules for the physical composition of complex genetic devices such as a multigenic constructs encoding synthetic metabolic pathways. Thus, standardized building blocks such as promoters, coding regions and terminators can be assembled into higher order modules following a discrete set of physical composition rules. Standard genetic modules can be reused for different engineering purposes, exchanged between laboratories and/or employed in combinatorial approaches, speeding up engineering. Furthermore, Modular Cloning props up the creation of databases and/or repositories of synthetic genetic elements and facilitates the implementation of computer-assisted DNA assembly design.

To facilitate engineering, we recently launched GBcloning, a comprehensive web-based tool devoted to Modular Cloning in Plants. The GBcloning tool makes use of the GoldenBraid2.0 assembly standard (an iterative Modular Cloning strategy), to build multigene constructs in binary plasmids. GBcloning comprises a database and a repository of exchangeable genetic elements as well as a set of software tools for assisting in the design of multigenic constructs.

Following with this endeavor, we are currently developing a new version of the GBcloning tool that expands its current capabilities. Among other features, the new version will bring more compatibility with other Modular Cloning methods and will include new domestication strategies to enable, among others, the conversion of intron-containing genomic sequences to the GB stantard. Most importantly, the new GBcloning will include functional descriptions of the genetic elements in its database. The ultimate challenge in (Plant) Synthetic Biology is to develop specifications and quantitative descriptions for each synthetic component, which should constitute the basis for the establishment of

functional (in addition to physical) composition rules. Ideally, functional composition rules should allow synthetic biological components to be reliably and predictably assembled into functional devices. Unfortunately, the complexity of the plant chassis converts the production of accurate and at the same time comprehensive descriptions for plant biological parts a nearly impossible task, and therefore certain compromise between accuracy and operability needs to be established. We are exploring ways to functionally describe (some) plant DNA elements by defining standard descriptive datasheets that incorporate quantitative and/or qualitative operative descriptions. In addition, we are producing and characterizing new genetic parts and assessing to what extent their standard functional descriptions (e.g. standardized measurements of transcriptional activity levels) can be used to predict the behavior of newly assembled modules.

O-29

Application of modular cloning for molecular pharming and plant metabolic engineering

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During the last years, modular cloning techniques have become widely used and several standards have been proposed and adopted by numerous labs. Within this COST Action, large attempts were made to promote this technology, develop new tools, train students and also promote standards which could be used by the community. Here we will describe a case study for expanding the tools for plastid transformation for metabolic engineering as well as for molecular farming purpose. With the viral entry inhibitor Griffithsin we were able to show the assembly of a plastid transformation vector from standardized parts and successful expression of the transgene in tobacco chloroplasts. Moreover, the technology platform can be expanded for multigene operons to express metabolic pathways. Examples how the COST network enabled collaborative research efforts by STSM, training schools and other community efforts will be given.

Session IV Posters

Future aspects and perspectives

P-36

Plastid biotechnology for high-level production of recombinant proteins

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The plastid genome (plastome) represents an attractive target for genetic engineering of crop plants. Although plastid transformation is technically more challenging than nuclear transformation, insertion of the transgene in the plastome offers several advantages (*e.g.,* high protein yields, precise integration of transgenes by homologous recombination, transgenes containment, etc.). Over the years, different biotechnological applications (metabolic engineering and molecular farming) of plastid transformation have been explored in our laboratory.

Recently, our efforts to produce recombinant proteins in plastids have been focused on thermostable biofuel enzymes, the endoglucanase from *Sulfolobus solfataricus,* β-glucosidase from *Pyrococcus furiosus* and the endo-β-1,4-xylanase from *Alyciclobacillus acidocaldarius* (courtesy of Dr. M. Moracci, CNR-IBBR UOS Napoli). Transgene expression was controlled by the strong plastid ribosomal RNA promoter (Prrn) and various 5' regulatory sequences (5'-UTR from phage T7 gene 10, 5'-UTR and 42 N-terminal nucleotides of the plastid *atpB* or *rbcL* genes). Further, a C-terminal Flag sequence was added for protein detection. Recombinant vectors were used for biolistic transformation of tobacco plants. Western analyses using anti-flag antibodies detected variable protein yields for the different cellulolytic enzymes. The highest protein yield (about 40-50 % of total soluble proteins) was obtained with the endo-β-1,4-xylanase. Preliminary enzimatic assay measured a comparable activity between plant-based and *E. coli*-based xylanase. Transplastomic plants producing the endoglucanase enzyme showed phenotypic alterations, such as pigment deficiency in leaves and growth retardation. Similar pleiotropic effects were also detected in transplastomic tobacco plants which expressed high level (up to 8 % TSP) of $Pr55^{gag}$ polyprotein, the major structural protein complex of human immunodeficiency virus (HIV-1). Such effects were due to the binding of $Pr55^{gag}$ polyprotein to thylakoids.

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Transgenic Res, doi: 10.1007/s11248-014-9845-5.

P-37

Potentially cytotoxic side effects of essential oils with good antibacterial activity on animal cell line

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Essential oils (EOs) derived from aromatic medicinal plants have been reported to exhibit a wide range of biological activities, with exceptionally good antimicrobial effects. Since bacterial contamination of the uterine lumen after parturition is common in dairy cattle, and uterine disease is an important cause of infertility, EOs may have potentials in the control of it, used as an active substance in intrauterine preparations for veterinary use. However, the use of EOs as antimicrobial agents is not only limited by their effective concentrations *in vitro*, but also the maximum dosage that can be administered without toxic side effects. That means that EOs may exert cytotoxic effects to tissue cells at concentrations which do not yet show an antibacterial effect. In the present work we used cell culture systems in order to predict EOs toxicity to mammalian cells *in vivo*.

Our research was focused on the EOs of different medicinal plants from our region (9 species). Triage plant products were performed by examination of chemical composition and antibacterial activity tests. EOs from savory, thyme, peppermint and some EOs constituents carvacrol, menthol, eugenol and thymol, were the most effective antibacterial substances against pathogens from animal utery, isolates (field strains) and bacterial strains obtained from ATCC, and they were tested for cytotoxic activity- influence on cell viability *in vitro*.
In this study, for cytotoxicity evaluations we used MDBK cell line which was derived from a kidney of an apparently normal adult steer, morphology epithelial. The cells were exposed to media containing different concentrations of EOs and constituents for 3, 6, 12 and 24 hours, after which the cell survival was evaluated by MTT assay.
The extracts from plants species and their main components exhibited different activity against the MDBK cell line. Among these, the thymol showed high cytotoxic activity with mean IC_{50} values of 0.49 and 0.42 µL(mg)/mL, during 12h and 24h exposure, respectively. Moderate cytotoxicity showed EO from thyme and carvacrol for all investiagated

exposure times ($0,5<IC_{50}<1$ µL(mg)/mL). The EOs from peppermint and savory presented least toxic side effects, with mean IC_{50} ranging from 11.9 to 2.62 and 1.56 to 1.02 µL(mg)/mL, respectively, like eugenol (2.44–1.12µL(mg)/mL) and menthol (10.37-1.22µL(mg)/mL). Dose-dependent cytotoxicity of tested substances towards MDBK cell line was confirmed, like time-dependent cytotoxicity on MDBK cells after 3h, 6h, 12h and 24 h exposure.

The results obtained have shown that EOs from savory, peppermint and EOs constituting menthol and eugenol were less toxic against MDBK cell lines, and their IC_{50} values were several times higher than MICs values of these substances. Considering antimicrobial potential and anti-cytotoxicity activity, safety ratio, overall activity of EOs can be ranged as follows from the most potent: *Satureja montana* L. > *Mentha x piperita* L. > *Thymus vulgaris*. These results undoubtedly validate the common use of these plant extracts in traditional and official medicine, as well as a potent source of natural medical substances. However, further research is necessary to confirm these results and assess the toxicity and the therapeutic effect of selected substances *in vivo*.

Keywords: essential oils; cytotoxicity; MTT; MDBK cell line

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P-38

Screening of plant metabolite libraries for the identification of innovative Hsp90 inhibitors

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Small molecules synthesized by plant kingdom can be considered evolutionary-chosen "privileged structures", since they have evolved in a natural selection process to achieve optimal interactions with biological macromolecules. Consistent with this notion, plant-derived molecules have shown an extraordinary potential as modulators of proteins functions and have been largely studied to uncover their specific molecular targets and their potential role in interfering with signaling pathways involved in human diseases. Currently, many plant molecules or their derivatives are included into pharmaceuticals present in the market, are under advanced clinical trials or are inspiring new leads for drug discovery.
Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone assisting the proper protein folding and assembly, and targeting misfolded proteins to the proteolytic degradation. Inhibition of the Hsp90 activity incapacitates simultaneously multiple client proteins, resulting in a blockade of multiple signaling pathways and, ultimately, providing a combinatorial attack to cellular oncogenic processes. Because of the potential therapeutic use in multiple cancer indications, Hsp90 has emerged as an exciting new target for the development of antitumor agents [2]. In an effort to discover new small molecules able to affect the Hsp90 activities, we developed a screening approach consisting of Surface Plasmon Resonance measurements, *in vitro* enzymatic tests and cellular assays. This approach was used to screen small plant-compound libraries, leading to the identification of some new potential Hsp90 inhibitors. Most promising candidates were also subjected to mass-spectrometry-based and computation-based structural studies, allowing to describe their molecular mechanism of action [3-6].

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P-39
Hydrophobin fusion facilitated production of high-value proteins in plants and BY-2 suspension cells

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The most critical bottleneck of Plant Molecular Farming is the downstream processing and purification of recombinant proteins. Hydrophobins (HFB) are small globular proteins from filamentous fungi having surface adhesion and amphipathic properties. In fungi, the HFBs have a broad range of biological functions: coat the hypha and spores, improve dispersion, provide surface adhesion and facilitate penetration of water air interfaces by decreasing surface tension. The biological roles of HFBs have inspired a multitude of potential uses in biotechnology from structure enhancing food additives to coating of sensors and nanoparticles. In addition, HFBs can be utilized as purification tags, enabling efficient capture of target proteins using a surfactant-based aqueous two-phase system (ATPS). Plants and plant cell cultures provide an excellent platform for manufacturing of HFB fusion molecules since they are capable of correctly folding hydrophobins and corresponding fusion partners. Until now, the HFB fusion technology has mainly focused on *Trichoderma reesei* HFB1 leaving the large diversity of other HFBs unexplored. Here we report plant based expression of several novel HFB tags fused to GFP. The established library of HFB tags was further used to engineer fusion proteins with human transferrin, human interleukin-22 and *Staphylococcus aureus* Protein A. All target molecules were fused to hydrophobin tags and expressed transiently in *Nicotiana benthamiana* and in transgenic tobacco BY-2 cells. The hydrophobin fusion tags moderately improved accumulation levels of all targets and allowed efficient purification by ATPS. In addition, protein production in standard stirred tank bioreactors was studied. BY-2 suspension cells allowed straightforward scalability and easy adherence to GMP. Also the downstream processing of the culture broth by high-pressure homogenization, filtering and ATPS was compatible to industrial scale processes. The demonstrated BY-2 expression platform combined to ATPS process offers an exciting alternative for recombinant protein production and purification

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