Challenges and prospects in PNP metabolic engineering and production

PlantEngine
COST Action FA1006

Final Conference

15-17 April, 2015

Sorrento, Italy

Background and aim of the meeting

Over the last four years, COST Action FA1006 has established a pan-European network of researchers working on different aspects of **plant natural products** (PNPs) and **metabolic engineering**. With a large number of activities, the community has elaborated the *status quo* of PNP research and identified target areas for improvement of PNP metabolic engineering.

The **final conference** will represent a cross-section through all the activities and build a platform to showcase major achievements of *PlantEngine*.

According to the structure of the COST Action, consecutive scientific sessions will reflect the Working Group topics and highlight achievements of their respective activities. Additionally, an industry-focused session will map out the *status quo* of commercialization of PNP engineering and technologies. Current collaborative EU research projects will be presented and prospects for continuation of the successful network discussed.

Program

Wednesday, April 15

10:00 Registration & poster set-up

14:00 Opening & welcoming address
Antonella Leone, IT; Heribert Warzecha, DE; Paul Fraser, UK

Session I
Capturing secrets from nature
chairs: *Paul Fraser, UK* & *Gianfranco Diretto, IT*

14:30 Keynote lecture: Carotenoid biosynthesis B.C. and A.C. and genetic pathway engineering for optimization of carotenoid yields
Gerhard Sandmann, J.W. Goethe Universität, Frankfurt, DE

15:15 Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations
Marilise Nogueira, UK

15:35 Development of an automated gene discovery framework to highlight pivotal plant metabolic and genomic features
Oren Tzfadia, BE/IL

15:55 **Coffee break & poster viewing**

16:30 Biochemical characterization of novel CCDs from saffron
Sarah Frusciante, IT

16:50 Iridoid synthase activity is common among the plant progesterone 5β-reductase family
Jennifer Munkert, DE

17:10 Keynote lecture: Making new molecules
Anne Osbourn, John Innes Centre, UK

18:00 Poster Session

19:30 **Welcome buffet**

Thursday, April 16

Session II
Filling gaps in plant secondary metabolism and metabolic engineering
chairs: *Maria Angeles Pedreño, ES* & *Oren Tsfadia, BE/IL*

09:00 Keynote lecture: Discovery of aromatic prenyltransferase gene family and its application to metabolic engineering
Kazufumi Yazaki, Research Institute of Sustainable Humanosphere, Kyoto University, JP

09:45 Enhanced sequestering of sesquiterpenes by coexpression of pathway genes with ABC-transporter and LTP genes in *Nicotiana benthamiana*
Sander van der Krol, NL

10:05 A molecular toolbox to engineer the metabolic flux of the anticancer alkaloids from *Catharanthus roseus*
Mariana Sottomayor, PT

10:25 **Coffee break & poster viewing**

chairs: *Angelos K. Kanellis, GR* & *Lorena Almagro, ES*

11:00 Multiple copies of a simple MYB-binding site confer trans-regulation by flavonoid-related R2R3 MYBs
Richard Espley, NZ

11:20 Plant roots as a perfume? Overexpression of a MYB transcription factor leads to methyl anthranilate emission in *M. truncatula* hairy roots
Jacob Pollier, BE

11:40 Driving metabolic flux towards high content of abietane-type diterpenes in *Salvia sclarea* hairy roots
Antonella Leone, IT

12:00 Transcriptomics and metabolomics approaches towards better understanding of BIA biosynthesis in opium poppy
Turgay Unver, TR

12:20 Transcript profiling of jasmonate-elicited Taxus cells to identify new genes involved in taxane biosynthesis: a new gene encoding a b-phenylalanine-CoA ligase
Karla Ramírez-Estrada, ES

12:40 Detection of wild alleles to engineer ascorbic acid metabolic pathway in tomato
Manuela Rigano, IT

13:00 **Lunch**

chairs: *Efraim Lewinsohn, IL* & *Marilise Nogueira, UK*

14:00 Polyketide synthases responsible for 4-hydroxy-5-methylcoumarin biosynthesis in gerbera
Teemu H. Teeri, FI

14:20 Type III polyketide synthases from poison hemlock
Heiko Rischer, FI

14:40 Monoterpenol oxidative metabolism for the biosynthesis of bioactive compound
Daniele Werck-Reichhart, FR

15:00 TILLING analysis of a *Medicago truncatula* mutant collection for identification of CYP450s involved in triterpene saponin biosynthesis
Elisa Biazzi, IT

15:20 **Coffee break & poster viewing**

Session III
Toward industrial applications of plant secondary metabolites
chairs: *Harro Bouwmeester, NL* & *Manuela Rigano, IT*

16:00 Keynote lecture: Plant made recombinant pharmaceuticals – where is the field?
*Julian Ma, St. George's University of London, UK**

16:45 Ketocarotenoid-rich tomato powders incorporated into natural-matrix microspheres: stability and controlled release for fish feeding and biomedical applications
Carmen Socaciu, RO

17:05 Betulins as novel lead compounds for cancer
Kirsi-Marja Oksman-Caldentey, FI

17:25 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
Lorena Almagro, ES

17:45 Effect of the mycotoxin ophiobolin A on cell proliferation, cell viability and redox state on tobacco Bright Yellow-2 cells
Esther Novo-Uzal, ES

18:05 Plant tissue culture as tools of bioactive ingredients with cosmetic applications
Fabio Apone, Arterra Bioscience, IT

20:00 **Dinner**

Friday, April 18

Session IV
Future aspects and perspectives
(presentation of ongoing collaborative projects)
chairs: *Ludger Wessjohann, DE* & *Mariana Sottomayor, PT*

09:00 TriForC
Søren Bak, DK

09:20 From DISCOvery to products: A next generation pipeline for the sustainable generation of high-value plant products
Paul Fraser, UK

09:40 Transgenic vs non-transgenic metabolic engineering of carotenoids in tomato
Joseph Hirschberg, IL

10:10 Saffronomics: Transcriptomics-based dissection of the saffron stigma apocarotenoid pathway
Giovanni Giuliano, IT

10:40 A library of TALE-activated synthetic promoters: application for metabolic engineering in plants
Alain Tissier, ERA-CAPS, DE

11:00 **Coffee break & poster viewing**

11:30 Keynote lecture: Production of immunoglobulins in plants – challenges and solutions
*Victor Klimyuk, ICON Genetics, DE**

12:00 Refinement of the standards for genetic design in plant synthetic biology using the GoldenBraid format
Marta Vazquez, ES

12:20 Application of modular cloning for molecular pharming and plant metabolic engineering
Heribert Warzecha, DE

12:40 Closing remarks
Heribert Warzecha, DE

14:00 – 16:00 Final MC meeting; reporting & evaluation

*scheduling rearranged upon speakers' request

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Proceedings

Wednesday, April 15

Welcoming address

The meeting was opened by the MC Chair *Heribert Warzecha, DE*, Vice-chair *Paul Fraser, UK* and local organizer *Antonella Leone, IT* who extended their welcome to the participants representing diverse domains of PNP research.

Session I

Capturing secrets from nature

chairs: *Paul Fraser, UK* & *Gianfranco Diretto, IT*

In the inaugural scientific presentation, *Gerhard Sandmann, DE* mapped out the history of unraveling the biosynthesis of carotenoids. Designating cloning of crt genes in the 1990s as a milestone and a turning point of the endeavor, he went on to highlight its most significant achievements. Focusing on nutritionally and economically significant carotenoids (e.g., phytoene, zeaxanthin, fucoxanthin, astaxanthin), he emphasized the potential of metabolic engineering for augmentation of biosynthetic outputs, both quantitative (enhanced levels of carotenoids of established value) and qualitative (extension of know biosynthetic routes leading to novel structures and activities) in nature. In closing, the speaker drew the participants' attention to the revival of traditional biochemical approaches as complementary to the tools of post-cloning era biotechnology for comprehensive PNP research.

Questions from the audience:

Q: What is the biochemical nature of divergence in astaxanthin biosynthesis in different kingdoms?

A: The contrasting desaturation mechanisms characteristic of plants and bacteria.

Q: What is the limiting factor of *crt* overexpression in microorganisms?

A: The restricted storage capacity.

Marilise Nogueira, UK addressed the aspects of metabolite regulation and sequestration in the aftermath of overexpression of carotenogenic genes in tomato. The observed perturbations included altered tissue specificity of the pathway gene transcripts as well as changes in the steady state levels of metabolites in unrelated sectors of metabolism. Of particular interest was the increased accumulation of the plastid-localized lipid, monogalactodiacylglycerol and membranous subcellular structures concurrent with the carotenoid build-up. The cumulative image of the transgenic tomato fruit sub-chromoplast fractions thus obtained suggested that cellular structures can adapt to

facilitate sequestration of the newly formed secondary metabolites. While the presented findings were obtained as a result of the *PlantEngine* STSM, their implications with respect to novel pathway regulation mechanisms will be further explored (successful grant application).

Questions from the audience:

Q: Were any differences in the enzyme organization between the chloroplast and the chromoplast observed?

A: The comparison on enzymatic level was not performed; however, the corresponding gene/transcript profiles differed.

Q: Were levels of other lipids affected?

A: Yes; however, the analysis did not include sterols.

Challenges of maximizing computer and mathematical techniques for enhanced identification of key player genes in metabolic and biological pathways, as founded upon the platform of rapidly increasing availability of high-throughput experimental '-omics' datasets, were discussed by *Oren Tzfadia, BE*. He further expounded upon the proposed 'data-driven philosophy', enumerating its prerequisites and obstacles to be tackled on the way to its full implementation:

- ✓ reliable and accurate identification of biological features (based on structural and functional annotation of pathway elements) performed in a fully automated manner,
- ✓ inter-species comparative analysis of the identified features (and combinations thereof),
- ✓ implementation of parallel and distributed computing techniques featuring algorithms enabling processing of large amounts of data.

Demonstrating the applicability of relevant bioinformatic tools (e.g., the MORPH platform, http://bioinformatics.psb.ugent.be/webtools/morph/), the speaker introduced the audience to the current state of development of an automated cost-effective-high-throughput-gene-discovery framework to, based on data mining and smart integration of various sources of information, fill gaps in metabolic pathways and transfer information from model to non-model plant species.

Questions from the audience:

Q: Was 'going back' to bacterial operons considered when applying the postulated platform?

A: The question is worth pursuing, but the initial attempts seemed much like 'shooting in the dark'.

Q: Was the system applied to the analysis of hormonal pathways, difficult to predict based on co-expression investigation due to time-lags?

A: Such analysis necessitates a different approach and was not implemented in the proposed work.

In her talk on biochemical characterization of novel carotenoid cleavage dioxygenases (CCDs) from saffron stigmas, *Sarah Frusciante, IT* presented results dispelling the conflicting findings of previous reports on the committed enzyme of apocarotenoid biosynthesis leading to the formation of metabolites contributing to the color, taste and aroma of the valuable spice. RNA-Seq profiling of six different saffron stigma developmental stages, from immature to fully developed, yielded identification of seven *CCD* candidates. The activity of the encoded enzymes was investigated *in bacterio* (engineered, carotenogenic *E. coli* cells), *in planta* (maize kernels) and *in vitro* (determination of enzyme stereospecificity), ultimately leading to the conclusion that CCD2, one of the newly identified dioxygenases, was the dedicated enzyme of saffron apocarotenoid biosynthesis. The presented, *PlantEngine* STSM-derived, research results were published in the *Proceedings of the National Academy of Sciences of the United States of America* (*PNAS*).

Questions from the audience:

Q: Were any modeling experiments performed to determine what enzymatic domains/elements were responsible for the specificity of zeaxanthin cleavage by CCD2?

A: No, but the 7,8- cleavage is not unusual among CCDs.

Q: Since the localization studies proved CCD2 to be a cytosolic enzyme, is its substrate also cytosol-specific?

A: Yes, it is. It can be further glycosylated, however, and directed to the vacuole.

Jennifer Munkert, DE presented results of a comprehensive analysis of substrate specificity/promiscuity of the progesterone 5β-reductase (P5βR) enzyme family members characteristic of various plant species. While traditionally described as being involved in cardenolide biosynthesis, P5βR representatives boast alternative functions, acting as iridoid synthases (IS) most notably implicated in the biosynthesis of bioactive monoterpenoid indole alkaloids (MIAs). The aforementioned substrate-based investigation of a set of recombinant P5βRs from angiosperm plant species not known to produce iridoids shed more light on the apparent functional redundancies, leading to the conclusion that the IS activity was intrinsic to P5βR proteins, having evolved early in the process of angiosperm diversification. Besides their role in the biosynthesis of pharmaceutically significant iridoids and cardenolides, the wide substrate range of P5βRs, including small toxic molecules (e.g., methylvinylketone), thus suggesting their detoxification capacities, can be interpreted as the evolution of different physiological functions for different P5βR family representatives *in planta*. The presented results, obtained in course of the *PlantEngine* STSM, were published in *Molecular Plant* (Cell Press).

Questions from the audience:

Q: In light of the presented results, the nomenclature of P5βR enzymes seems obsolete. Are any prospective changes under way?

A: The issue was addressed; however, modifying the traditional nomenclature order might prove confusing.

In her keynote lecture, closing the day's proceedings, *Anne Osbourn, UK* highlighted the fact that the vast majority of plant metabolic diversity was as yet untapped, despite its huge potential value for humankind. She further suggested that the recent discovery that genes for the synthesis of different kinds of natural products were organized in clusters in plant genomes was now opening up opportunities for systematic mining for new pathways and chemistries. Providing relevant examples (as comprehensively reviewed in Nützmann & Osbourn, 2014; *Curr Opin Biotechnol* doi: 10.1016/j.copbio.2013.10.009), the speaker expounded upon the potential of improved understanding of the genomic organization of diverse types of specialized metabolic pathways for unraveling the mechanisms underpinning pathway and genome evolution, thus 'providing grist for the synthetic biology mill'.

Questions from the audience:

Q: Was the sliding window technique applied for identification of new clusters?

A: Yes, along with other approaches.

Q: Have any cluster-specific transcriptional factors been identified?

A: No clear answers have been attained yet.

Q: Do the clustered genes encode solely pathway enzymes? Have any transporter genes been identified?

A: So far, no cluster-related transporters have been determined as a result of the speaker's research endeavors. However, S. O'Connor reported on a cluster from *Catharantus roseus* including at least one.

Thursday, April 16

Session II

Filling gaps in plant secondary metabolism and metabolic engineering

chairs: *Maria Angeles Pedreño, ES* & *Oren Tsfadia, BE/IL*

Kazufumi Yazaki, JP initiated the second day of meeting proceedings. In the introductory part of his keynote talk, he highlighted the significance of prenylation as a biosynthetic transformation driving overarching increase in biological activity of specialized metabolites (e.g., flavonoids, coumarines and furanocoumarines). Supporting his claim with relevant examples, he delineated the on-going efforts

aimed at the discovery of genes encoding enzymatic representatives of the aromatic prenyltransferase family. He subsequently addressed the challenges of metabolic engineering involving prenylation of natural products in pursuit of superior and novel bioactivities, drawing the participants' attention to the aspects of transport engineering. In closing, the speaker emphasized the importance of further developments in the field of prenyltransferase research for prospective boost in quality of human health.

Questions from the audience:

Q: What is the source pathway for the prenyl chain? Would the engineered increase in MVA/MEP production drive prenyltransferase activity?

A: In case of flavonoids, MEP pathway provides the prenyl moiety. The metabolic engineering trials in question are in progress; their results, however, have been marginal so far.

Q: Have any aspects of transcriptional regulation of prenyltransferases been uncovered?

A: The transcriptional control mechanisms remain unknown.

Q: Does prenylation of specialized metabolites imply their targeting (as is the case for prenylated proteins)?

A: Prenylated PNPs are directed to the apoplast and subsequently transpoted out of the cell (excreted). When ingested, however, they remain within human cells for long periods.

Sander van der Krol, NL expounded upon the aspects of enhanced sesquiterpene sequestration driven by co-expression of pathway genes with those encoding transporter functions. Accordingly, the effect of two pleiotropic drug resistance (PDR) type membrane transporters and three lipid transfer proteins (LTP) from *Artemisia annua* on levels of free artemisinic and dehydroartemisynic acid (AA and DHAA), precursors of the antimalarial artemisinin, were tested to counteract their accumulation as biosynthetically inactive glycosides upon transient pathway gene transfer into the heterologous *Nicotiana benthamiana* system. Comprehensive investigation of individual transporter activity in a wide array of available combinations led to successful determination of the optimal permutation (pathway genes + PDR2 + LPT3), resulting in retrieval of AA and DHAA yields comparable to those characteristic of the original host plant. Admitting the lingering existence of yet unanswered questions, the speaker attributed the aforementioned progress towards ultimate elucidation of metabolite transport mechanisms to the work of young scientists.

Questions from the audience:

Q: Has the investigation of wax as the hydrophobic environment been considered in the context of transport?

A: The approach has been considered and will be attempted.

Highlighting the relevance of *PlantEngine* initiatives: STSMs (recipients, *Teresa Martínez-Cortés* and *Sarah Bettoncourt*; see also proceedings of WG3 meeting in Warsaw, 2014) and Training Schools (VIGS), *Mariana Sottomayor, PT* mapped out the on-going comprehensive research aimed at the ultimate elucidation of the biosynthesis of highly specialized, potent antineoplastic monoterpenoid indole alkaloids (MIAs) of *C. roseus*. Invoking the intricacies of the process in question – its complex sub-cellular as well as cell type-specific compartmentation implying multiple levels of metabolite traffic – she focused on the challenges of unravelling the mechanisms of transport as well as transcriptional control in MIA formation. In conclusion, she characterized the presented body of research as a stepping stone to the generation of a molecular toolbox enabling the design of successful metabolic engineering strategies for increased retrieval of the *C. roseus* anticancer alkaloids.

Questions from the audience:

Q: Have sugar transporters been taken into account as potentially involved in the excretion of MIAs from the vacuole to the cytosol?

A: Many strategies have been considered; their implementation was hampered, however, by the difficulties in the design of reliable assays for outward transport characterization.

chairs: *Angelos K. Kanellis, GR* & *Lorena Almagro, ES*

Richard Espley, NZ discussed the recent increase in research activity aimed at anthocyanins and their contribution to a healthy diet. He then specifically highlighted genetic engineering endeavors focused on the R2R3 MYB transcription factor, MYB10 as a regulatory element of anthocyanin accumulation in apple. The success of initial experiments in the native plant, resulting in fruit associated with a decrease in inflammation markers and changes in gut microbiota, as attested by animal trials, was further translated to alternative systems. The MYB10-driven transcriptional autoregulation, as manifested by binding its own promoter motif (R1), was further exploited (mutation of the motif in the red-fleshed apple germplasm, forming a minisatellite repeat unit, R6), resulting in generation of plants boasting elevated anthocyanin levels (pear and *Arabidopsis thaliana*) or altered athocyanic profiles (increased accumulation of delphinidin in tobacco and kiwi). In closing, the speaker postulated that the aforementioned naturally occurring motif provided a versatile tool to re-engineer novel MYB-regulated responses across a range of plant species.

Questions from the audience:

Q: Since the animal study indicated changes in bacterial gut flora, was there any evidence suggesting that anthocyanins might play a role in defense against pathogens?

A: The human trial is currently in progress. The effect is expected to be less pronounced than in mice.

Q: As the anthocyanin biosynthesis is tightly regulated, were any pleiotropic side-effects observed in the engineered plants, especially concerning stress response?

A: The possible stress tolerance effects are yet to be investigated.

Q: What was the taste of the engineered apples?

A: According to the consumer panel, the taste of the fruit was pleasant. Storage, however, posed a challenge.

Yet another MYB-driven metabolic process was expounded upon by *Jacob Pollier, BE*. Identified through transcript profiling of jasmonate-treated *Medicago truncatula* suspension cells and expressed in the hairy roots of the legume, the transcription factor spurred emission of the volatile compound, methyl anthranilate, conferring a fruity scent. The subsequent RNA-Seq analysis of the fragrant roots revealed upregulation of an anthranilate *O*-methyltransferase, whose promoter, however, upon further investigation, did not prove the direct target of the transcription factor in question. Extended probing of the RNA-Seq data led to the identification of a PLATZ repressor protein implicated in the MYB-activated negative feedback loop – a principle seemingly widespread in plant secondary metabolism and a likely strategy aimed at safeguarding plant integrity and protecting the plant form overaccumulation of particular bioactive specialized metabolites.

The facets of rational metabolic flux stimulation were invoked by *Antonella Leone, IT* in the context of current endeavors aimed at enhanced accumulation of the valuable, potentially antineoplastic, abietane diterpenoids in *Salvia sclarea* hairy roots. The experimental design included tuning the expression of genes controlling putative limiting biosynthetic steps (alone or in combination) in concert with the silencing of a gene relevant to a lateral competing metabolic route, as well as over-expression of relevant transcription factors. Highlighting the substantial contribution to the undertaken research project rendered by the *PlantEngine* STSM, the speaker drew the participants' attention to the successful enhancement of the valuable metabolite content in the engineered hairy roots, describing the aforementioned complementary approaches as strategies paving the way to the rational design of a production platform yielding reliable amounts of abietane diterpenoids and their future commercialization.

Questions from the audience:

Q: Did the described methyl jasmonate elicitation trials influence the hairy root biomass?

A: Yes, they did. That's why it is preferable to apply metabolic engineering strategies.

Q: Do other *Salvia spp*. produce aethiopione (the abietane diterpenoid of the most pronounced cytotoxic activity)?

A: Accumulation of the metabolite has not been described in alternative species.

Turgay Unver, TR expounded upon the multidisciplinary strategies employed in tackling the challenges of delineation of the complex and highly regulated biosynthetic route affording formation of benzylisoquinoline alkaloids (BIAs) in opium poppy. The applied versatile approaches comprised genome-wide microarray expression analyses of high-yielding cultivars followed by selective fine-tuning of gene expression (over-expression vs. silencing) in diverse plant tissues. The genetically manipulated plants were further subjected to large-scale metabolomic (HPLC/ToF-MS) and transcriptomic analyses, while microRNA regulation of BIA biosynthesis was studied by means of next generation sequencing (NGS) tools.

Describing plant cell cultures as eco-friendly biofactories of pharmaceutically significant natural products and platforms facilitating application of innovative research strategies to advance the understanding of secondary metabolism at large, *Karla Ramírez-Estrada, ES* presented the results of a genome-wide expression analysis performed upon jasmonate-elicited *Taxus baccata* cell cultures aimed at definitive mapping out of as yet ambiguous biosynthetic steps affording formation of the potent antineoplastic agent, taxol. Successful identification of *TB768* as the gene encoding acyl-CoA ligase relevant to the formation of taxol lateral chain, rendering the metabolite bioactive, led the speaker to conclude that transcriptomic profiling, as monitored by cDNA-AFLP, coupled with *in silico* capabilities of relevant bioinformatic techniques constituted a cutting-edge set of tools for the identification of new genes involved in metabolism of valuable specialized compounds. The presented results, obtained in course of the *PlantEngine* STSM, were published in *Plant Biotechnology Journal*.

Questions from the audience:

Q: Why was this particular gene selected for further investigation from the 15 putative candidates obtained in course of the profiling?

A: On the one hand, the bioinformatics data suggested *TB768* to be a superior candidate; on the other, substrate availability played a role in further functional characterization of the potential taxol biosynthetic genes.

Maria Rigano, IT addressed the challenges of unravelling the multi-level complexities of ascorbic acid (AsA) biosynthesis in tomato, invoking superior antioxidative properties of the metabolite associated with a reduced risk of cancer, inflammation and cardiovascular diseases. Comprehensive dissection of the metabolic network in question was aimed at identification of loci expected to contribute to the entire pathway, their genome distribution and possible redundancy in terms of multi-copy genes, and performed through integration of transcriptomic data with genome-scale resources by means of relevant bioinformatic tools. The initial results were further corroborated by metabolic studies. In

closing, the speaker highlighted the potential of future transfer of the identified favorable wild alleles into the cultivation background through breeding or metabolic engineering.

Questions from the audience:

Q: How much can the AsA levels be elevated through pathway engineering before the negative feedback kicks in?

A: There are so many aspects and cross-overs with other pathways to consider that it is difficult to predict if there is a limit and what it ultimately would be.

chairs: *Efraim Lewinsohn, IL* & *Marilise Nogueira, UK*

Characterization of polyketide synthases responsible for 4-hydroxy-5-methylcoumarin (HMC) biosynthesis in gerbera was the topic of the experimental work presented by *Teemu Teeri, FI*. He juxtaposed the previously described G2PS1, a chalcone synthase-like polyketide synthase implicated in the synthesis of triacetolactone (a putative precursor of gerberine and parasorboside, two bitter-tasting glucosidic compounds involved, along with HMC, in plant defense against pathogens and herbivores), to its newly characterized, HMC-generating counterparts, G2PS2 and G2PS3. The corresponding genes were heterologously expressed in *N. benthamiana*, resulting in accumulation of an unreduced HMC precursor, while their ectopic expression in gerbera led to the formation of the coumarine of interest in tissues originally lacking the compound.

Questions from the audience:

Q: Were any metabolites in glucosidic form detected in transgenic tobacco plants?

A: No, as *G2PS2* and *G2PS3* were co-expressed with a glucosidase gene.

Heiko Richer, FI, after pointing out the significance of coniine in a historical context (poisoning of Socrates), focused on the unique biosynthetic process leading to the formation of the compound and related alkaloids in hemlock. In contrast to other piperidines, originating directly from amino acids, hemlock alkaloids are derived from acetate units via a polyketide, implying the biosynthetic involvement of a type III polyketide synthase (PKS). The aforementioned considerations spurred the comprehensive screening for PKS genes in *Conium maculatum*, resulting in the isolation and characterization of the most promising candidates.

Questions from the audience:

Q: Is an aminotransferase involved in the biosynthesis of coniine?

A: Yes. While PKS catalyzes the initial step of the pathway, the consecutive ones would involve aminotransferase activity. While a relevant enzyme has been characterized, the corresponding gene is still unknown.

Results of the on-going, multifaceted investigation of the CYP76 enzyme family were presented by *Danièle Werck-Reichhart, FR*. After pinpointing the outcomes of *PlantEngine* collaboration aimed at functional characterization of the cytochrome 76C subfamily representative from *A. thaliana*, CYP76C1 and leading to the delineation of its involvement in plant defense against florivore insects, she went on to describe the concomitant development of new tools for the generation of fragrant and aromatic metabolites (e.g., lilac compounds) and engineered production of biopesticides and insect deterrents. The speaker further emphasized the recent demonstration of the involvement of CYP76 enzymes in multiple geraniol oxidation steps in *C. roseus* secoiridoid pathway of MIA biosynthesis as well as in the generation of terpenic aroma in wine and fruit.

Elisa Biazzi, IT, the last speaker of the session, presented the outcomes of TILLING analysis of *M. truncatula* mutant collection aimed at identification of CYP450s involved in triterpene saponin biosynthesis. Functional characterization of the most promising gene candidate, following its heterologous expression in yeast, confirmed successful application of the aforementioned approach. Comprehensive substrate affinity tests are currently in progress, as are phylogenetic studies. In closing, the speaker emphasized the potential of the undertaken experimental efforts for future breeding and engineering strategies resulting in generation of high-yielding legumes and retrieval of reliable amounts of the valuable saponins, relevant in pharmaceutical, agrochemical, food and cosmetic industry branches.

Session III

Toward industrial applications of plant secondary metabolites

chairs: *Harro Bouwmeester, NL* & *Manuela Rigano, IT*

In his keynote address, *Julian Ma, UK*, the Founding President of the International Society for Plant Molecular Farming (http://www.societyformolecularfarming.org/), delineated the objectives and priorities of the nascent organization established in 2014. Putting the research field into historical perspective, he enumerated its mile-stone achievements: from the first *Nature* cover story on *in planta* generation of monoclonal antibodies (mAb), through the account on the development of Elelyso™ (recombinant glucocerebrosidase) – the first plant-made pharmaceutical to win approval by the U.S. Food and Drug Administration, to the successful experimental use of ZMapp™ (a cocktail of three chimeric mAbs, now in clinical trial) during the 2014 West African Ebola outbreak. He then addressed the future challenges of molecular farming, enumerating next-generation target products and pointing out the ever-present issues with the regulators as well as the growing necessity of the transfer of relevant technologies to the developing countries. Extending an invitation to the 2nd

Society meeting in Ghent, Belgium (25-27.05.2016), the speaker encouraged the audience to join and support the ISPMF.

Questions from the audience:

Q: There was no news on the ZMapp™ mAbs being plant-made in the popular press; why?

A: It was a conscious choice to give the story a 'wonder drug spin' and not draw 'GMO-haters'.

Q: What is the strategy of the Society to promote the largely academic research field in the private sector?

A: That is the area in which keeping the community together (ISPMF + *PlantEngine*) becomes paramount, facilitating concerted lobbying for funds.

Q: Is the near 100% purity of mAb preparations necessary for their therapeutic approval?

A: While not in case of Abs, purity might become an issue with other products (e.g., vaccines, adjuvants, etc.). On the other hand, the 'impurities' could impart new bio-advantages to the plant-derived preparations.

Carmen Socaciu, RO discussed the emerging technologies in microencapsulation for improved stability, bioavailability and controlled release of bioactive compounds. She supported her claims by presentation of relevant results pertaining to the stability and release parameters of an array of carotenoids and polyphenols in/from encapsulated samples derived from various metabolically engineered tomatoes. While high-ketocarotenoid microspheres showed superior stability in saline water supporting their application as feed supplements in fish feeding, those rich in astaxanthin were recommended as dietary supplements facilitating controlled release of the antioxidant in gastric and intestinal environment. Concluding her talk, the speaker invited the audience to the upcoming 8th International Congress on Pigments in Food in Cluj-Napoca, Romania (28.06-01.07.2016).

Questions from the audience:

Q: Were the toxic activities of highly concentrated flavonoids a concern?

A: No, as the final phenylpropanoid levels were rarely higher than those natural to wild-type tomato; however, no toxicological studies were conducted.

Re-utilization of betulin – a major constituent of the white birch outer bark, currently considered a low-value forest industry waste product of no economic significance – was the topic expounded upon by *Kirsi-Marja Oksman-Caldentey, FI*. The presented experimental procedure comprised synthetic generation of an array of betuline-derived compounds, their subsequent multi-level testing for anticancer activity and further structural modification through *in vitro* biotransformation in plant cell systems. The comprehensive screen yielded several promising candidates showing specific anti-invasive properties in mechanism of action and structure-activity relationship studies against an aggressive prostate cancer cell line.

Questions from the audience:

Q: As betulin and its derivatives are highly hydrophobic, how was the problem of solubility addressed in bioactivity testing?

A: DMSO was adopted as the primary solvent; some compounds, however, would still not solve.

Q: Has the application of cyclodextrins been considered to increase botulin solubility in plant systems?

A: Yes; various cyclodextrin ratios were utilized.

Q: Why was biotransformation undertaken instead of semi-synthesis?

A: Complementary application of both approaches has been considered; some modifications, however, are problematic in a synthetic setting.

Q: What is the molecular target of betulins in the cancer cells?

A: The question is currently being addressed.

Concerted application of various known trigger molecules of plant secondary metabolism was discussed by *Lorena Almagro, ES*. After delineating their postulated individual modes of action, she went on to describe the synergistic effect of diadenosine triphosphate (Ap_3A) and cyclodextrins (CDs) on the accumulation of *trans*-resveratrol resulting in considerably enhanced retrieval of the bioactive stilbenoid from the medium of *Vitis vinifera* cultured cells. However, while both elecitors specifically induced expression of phenylpropanoid pathway genes (*PAL*, *C4H* and *4CL*) as well as that encoding stilbene synthase 1 when supplemented individually, no synergistic transcriptional up-regulation effect spurred by Ap_3A-CDs combination could be established. The presented research outcomes were obtained as a result of the *PlantEngine* STSM.

Questions from the audience:

Q: Has the influence of Ap_3A on secondary metabolism been tested in other plant cell lines?

A: Its impact on cell growth is currently investigated. Future elicitation studies are foreseen.

Q: CDs were applied in the study in very high concentrations (up to 50 mM); why?

A: As resveratrol is toxic for plant cells, CDs additionally act as sequestration agents facilitating secretion and accumulation of the stilbenoid in the medium.

Q: Are CDs truly elicitors or simply 'traps' for the target metabolite, then?

A: They seem to fulfill both functions.

Ester Novo-Uzal, ES expounded upon the effects of ophobiolin A, a known mycotoxin, on proliferation, viability and redox state of tobacco Bright Yellow-2 (BY-2) cells. In contrast with previously reported programmed cell death (PCD) mechanisms, the ophobiolin A-triggered PCD of BY-2 cells was not mediated by an early overproduction of reactive oxygen species (ROS), neither did it involve alterations in the ascorbate-glutathione (ASC-GSH) metabolism. Moreover, while lower

concentrations (<10 µM) of the investigated mycotoxin did not affect cell viability, they triggered a reversible S/G2 cell cycle arrest, impeded the activity of poly(ADP-ribose) polymerases (PARPs) – nuclear enzymes involved in DNA repair and interfered with GSH metabolism. The presented results were obtained in course of the *PlantEngine* STSM.

Questions from the audience:

Q: Were any conclusions as to why fungi produce ophobiolin A drawn in course of the presented research project?

A: The mycotoxin facilitates feeding on the plant; its exact mode of action, however, is still unknown.

In the final talk of the day, *Fabio Apone, IT* highlighted the superior features of plant cell cultures as optimal systems facilitating generation of a plethora of active ingredients relevant for cosmetic applications. Alternative production platforms, such as hairy root cultures, were further discussed. The speaker supported his claims introducing various cosmetic products developed by Arterra Bioscience (http://www.arterrabio.it/).

Questions from the audience:

Q: When does a cosmetic become a pharmaceutical?

A: A pharmaceutical is usually defined as a pure compound of specific, validated activity. Cosmetics can be mixtures of compounds.

Friday, April 17

Session IV

Future aspects and perspectives
(presentation of ongoing collaborative projects)

chairs: *Ludger Wessjohann, DE* & *Mariana Sottomayor, PT*

The last day of meeting proceedings was initiated by *Søren Bak, DK*. He introduced the audience to the EU-funded consortium, *TriForC* (Triterpenes For Commercialization; http://triforc.eu/), highlighting its far-reaching goal: the development of a pipeline for the discovery, sustainable production and commercial utilization of known and novel high-value triterpenes with new or superior biological activities. He further enumerated specific challenges of the European industry to be addressed by the cooperative project in question:

- ✓ sustainable access to triterpene-plant source material as facilitated by newly established platforms allowing bioreactor-based production of triterpenes from plants that are endangered or difficult to cultivate,

- ✓ overcoming bottlenecks in triterpene metabolic engineering through utilization of cutting-edge gene mining concepts, creation of a genetic toolbox and establishment of a synthetic biology platform for the versatile production of designer triterpenes in organisms amenable to bioindustry-scale cultivation,
- ✓ optimal use of triterpene-producing plant biomass through conscious assessment of different production sources and investigation of downstream processing, separation and biorefinery possibilities.

Questions from the audience:

Q: Has implementation of the postulated pipeline afforded detection of any interesting biosynthetic patterns?

A: Yes; hyperglycosylation has emerged as the most prominent pattern observed so far.

DISCO (High Value Plant Products – From DISCOvery to final product; http://disco-fp7.eu/), the new academic/industry alliance bringing together pan-European and IPCP partners with complimentary multidisciplinary expertise, was introduced by *Paul Fraser, UK*. He emphasized the industry-driven research and demonstration activities of *DISCO* that will:

- ✓ exploit existing and evolving biodiversity in Solanaceae and Iridaceae to perform bioprospecting with state-of-the-art metabolomics approaches for the target molecules of interest (carotenoids, other terpenoids and tropane alkaloids),
- ✓ 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 downstream processes and integrative biorefining strategies for co-product and biomass utilization that reduce environmental impact,
- ✓ demonstrate production feasibility and product efficacy beyond the present state of the art.

Questions from the audience:

Q: What types of bioactivity assays are to be applied in course of the project?

A: *In vitro* and *in vivo* testing is foreseen (mostly based on colorimetric methods). The ultimate aim, however, is to usher the developed products into human trials.

Expounding upon the objectives and activities of the aforementioned *DISCO* initiative, *Joseph Hirschberg, IL* drew the participants' attention to the persistently unfavorable public perception of

genetically modified (GM) plants as sources of nutritional products. He further focused on non-GM metabolic engineering efforts (supported by *DISCO*), aimed at development of tomato varieties affording enhanced retrieval of phytoene and phytofluene (P&P) – carotenoids of pronounced anti-oxidative activity. He then juxtaposed the successful application of the alternative engineering strategy and those relying on transgenic interference for optimization of secondary metabolite yields and bioactivities.

Questions from the audience:

Q: What is the speaker's opinion on the potential of the new powerful tool of whole genome transfer proposed by Ralph Bock?

A: While the proposed strategy is applicable only to closely related plant species, the missing/weak link in the '-omics' approach overall seems to be 'phenomics' (phenotype analysis); that is the domain warranting further in-depth investigation.

Giovanni Giuliano, IT, invoking the experimental results presented by *Sarah Frusciante, IT* during the inaugural session of the meeting, put them into a broader perspective of global pathway elucidation. Dubbed *Saffronomics* and supported by the COST Action FA1101 (http://www.saffronomics.org/), the research endeavor consisted in deep transcriptome sequencing of *Crocus sativus* stigma at various stages of development and resulted in identification of candidate biosynthetic and transporter genes of the entire metabolic route leading to the valuable saffron apocarotenoids, from the initial cleavage of zeaxanthin to the cytoplasmic glycosylation of crocetin and the vacuolar sequestration of glycosylated crocins. In closing, the speaker invited the participants to the Gordon Research Conference and Seminar, *Carotenoids* in Tuscany, Italy (22-27.05.2016).

Questions from the audience:

Q: From among the identified MATE and ABC transporters, which were primarily implicated in the traffic of apocarrotenoids?

A: ABC transporters were the primary vehicles for apocarotenoids while their MATE counterparts conveyed flavonoids and other metabolites.

Q: Which ABC transporters were involved specifically?

A: They are currently being patented, so the information could not be directly delivered.

Q: Were the observed transport events a reflection of affinity or rather concentration of compounds in the investigated mixture?

A: The metabolite traffic in question was affinity-driven (e.g., *trans*- isomers were not transported at all).

Q: Is there, consequently, an alternative transport system for *trans*- apocarotenoids?

A: The latest speculation posits that *cis*- metabolites are converted to their *trans*- isomers already in the vacuole.

The challenges of coordinate expression of pathway genes in targeted tissues of stable transgenic plants as a prerequisite of effective engineering of complex metabolic networks were addressed by *Alain Tissier, DE*. The proposed solution consisted in the development of a library of 48 synthetic promoters controlled by a single designer Transcription Activator-Like Effector (TALE) through application of Golden Gate cloning technology. Utility of the assembled promoters for metabolic engineering was initially confirmed in transient assays facilitating expression of genes involved in the formation of plant diterpenoids and further investigated through subsequent generation of stable *N. tabacum* and *A. thaliana* transformants of divergent diterpenoid gene expression patterns (glandular trichomes vs. epidermis). The speaker emphasized the support of the ERA-CAPS funded project, *HIP* (Homeostasis of Isoprenoids in Plants: understanding compartmentalization, flux and transport of isoprenoids in glandular trichomes for non-crop and crop species; http://www.eracaps.org/joint-calls/era-caps-funded-projects/era-caps-first-call-%E2%80%93-2012/homeostasis-isoprenoids-plants) for the delineated, on-going experimental work.

Questions from the audience:

Q: Have any proteomic analyses or testing of RNA-binding effects accompanied the undertaken trials?

A: No, but the involved researchers are open to suggestions.

In his talk (originally due in Session III, Toward industrial applications of plant secondary metabolites), *Victor Klimyuk, DE* introduced the audience to the innovative plant engineering technologies implemented by ICON Genetics (http://www.icongenetics.com/html/home.htm). Expounding upon the ICON portfolio of Transgene Operating Systems (TOS)™, he emphasized their applicability for efficient production and glyco-engineering of mAbs as well as cost-effective plastid transformation (novel EtOH-inducible expression system). In closing, the speaker invited the participants to future cooperation.

Questions from the audience:

Q: Is the EtOH-inducible system prone to yeast growth/contamination?

A: The contamination risk exists, but, according to the on-going tests, is negligible.

Q: Are any Golden Gate-compatible promoters being developed?

A: The process of promoter diversification is under way.

Q: What sorts of downstream processing strategies are being applied in the plant-derived mAb production?

A: The utilized approaches are compatible with those used for mAb retrieval from mammalian cell systems.

Q: How is protein binding by plant polyphenolics addressed?

A: The problem is manageable (e.g., through pH manipulation).

Q: Are the issues of localization/compartmentation considered?

A: For each new target product, a set of signal peptides is tested and the most efficient system selected for further implementation.

Highlighting the increasing relevance of synthetic biology-inspired modular DNA assembly methods for plant metabolic engineering, *Marta Vázquez-Vilar, ES* familiarized the audience with the recent developments and updates of the *PlantEngine*-supported GoldenBraid standardized iterative modular cloning platform (https://gbcloning.upv.es/). New features of the proposed GB3.0 system include alternative domestication strategies enabling conversion of intron-containing genomic sequences to the GB standard and, most importantly, functional description of the GB-database genetic elements and devices (standard datasheets incorporating quantitative and/or qualitative operative descriptions possibly facilitating predictive modeling of newly assembled modules).

Questions from the audience:

Q: Is GB compatible with the MoClo system?

A: Yes, it is.

Q: Does the system have many users?

A: GB starter kits are requested every week – the number of users is growing steadily.

Heribert Warzecha, DE, the Chair of the COST Action FA1006 *PlantEngine*, was the final speaker of the meeting. He further emphasized the relevance of the aforementioned GoldenBraid system discussing its successful tailoring to the prerequisites of plastid transformation, ultimately affording the all-encompassing applicability of GBcloning in plant metabolic engineering. He then drew the participants' attention to the upcoming *New Phytologist* report: *Standards for Plant Synthetic Biology: a common syntax for exchange of DNA parts*. In the second part of his talk, the Chair summarized the activities and achievements of *PlantEngine*, placing a particular emphasis on the Action efforts promoting and championing young researchers. In closing, he encouraged the meeting participants to take full advantage of the *PlantEngine* website, continually featuring relevant updates and developments in the field, as a networking tool facilitating further rewarding cooperation.