Plant Metabolic Engineering for High Value Products

WG1 & WG2 Meeting February 28 – March 1, 2013 Porto, Portugal

Programme

Thursday **February 28**

9:00 Welcome and aims of the meeting
Marianna Sottomayor, IBMC/FCUP, Portugal

9:05 Aims and deliverables of *PlantEngine*
Heribert Warzecha, TU Darmstadt, Germany

WG1 *Status quo* and roadmap

9:20 Update on WG1
Paul Fraser, RHU, UK

9:35 Isoprenoids. *Status quo* of pathway elucidation and metabolic engineering
Paul Fraser, RHU, UK

9:50 Polyphenols. *Status quo* of pathway elucidation and metabolic engineering
Stefan Martens, EMF, Italy

10:05 Benzylisoquinoline alkaloid biosynthesis
– a brave new world built on a century of discovery
Peter Facchini, University of Calgary, Canada

10:20 Biocatalytic approaches to industrial plant natural products
Ludger Wessjohann, Leibniz IPB, Germany

10:35 Discussion

11:00 Coffee break

WG2 Molecular Tools:
North American experience in deep sequencing and metabolomics

11:30 The plant alkaloid factory: should we modernize or move to a new location?
Peter Facchini, University of Calgary, Canada

12:00 Advancing drug development and chemical production platforms in plants
Joe Chappell, University of Kentucky, USA

12:30 Discussion

13:00 Lunch

WG2 Molecular Tools:
PNP bottlenecks

14:30 Major bottlenecks of plant natural product research
Sarah O'Connor, JIC, UK

14:50 Molecular breeding for improvement of plant natural products
Ian A. Graham, University of York, UK

15:10 Major bottlenecks of PNP research and translation to industrial scale
Stefan Schillberg, Fraunhofer, Germany

15:30	Discussion
15:50	Coffee break

WG2 Molecular Tools:
Synthetic biology/metabolic engineering tools

16:15	Reconstitution of plant natural product pathways in microorganisms *Sarah O'Connor, JIC, UK*
16:30	A molecular toolbox for plant metabolic engineering: the Golden Braid technology *Diego Orzaez, IBMCP, Spain*
16:45	A molecular toolbox for plant metabolic engineering: the Golden Gate technology *Sylvestre Marillonet, Leibniz IPB, Germany*
17:00	WG2 Molecular Tools – Group discussions
18:30	Port wine tasting – informal discussions
20:00	Dinner

Friday **March 1**

9:00	WG2 Molecular Tools – Group discussions
10:30	Coffee break
11:00	WG2 Molecular Tools – Final discussion
12:30	Lunch

Participants

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Proceedings

Thursday, February 28

WG1 *Status quo* and roadmap

The meeting was opened by the host and local organizer, *Mariana Sottomayor, PT*, who extended her welcome to the participants from diverse fields.
The welcoming address was followed by a short introduction to COST and the specific goals of COST Action FA1006, *PlantEngine*, presented by the MC Chair, *Heribert Warzecha, DE*.

After presenting a concise update on the activities of WG1: *Status quo* and road map, predominantly featuring the identified shortcomings of PNP research (e.g., lack of comprehensive knowledge on metabolite flux and cell compartmentation, deficiencies in data analysis & integration, shortage of chemical standard libraries and want of multifaceted systems models) to be further addressed in course of the WG2: Molecular tools meeting, *Paul Fraser, UK*, Vice Chair of the COST Action and coordinator of METAPRO consortium, focused on the presentation of the current trends and developments in isoprenoid research. Discussing the literature highlights in the field (mentioning the contributions of *Harro Bouwmeester, NL* & *Joe Chappell, US*, in particular), he presented encouraging reports on metabolic engineering of carotenoid biosynthesis in solanaceous plants, concluding that most biosynthetic pathways are amenable to engineering and optimization. He further drew the participants' attention to the possible obstacles that might arise in course of engineering attempts, naming the interference with cellular processes due to the disturbed primary vs. secondary metabolism equilibrium and sequestration of target metabolites within sink organs, among others. His further recommendations included focussing on a metabolomic approach aiming at modelling and construction of correlation networks, PNP pathway elucidation of non-model plants and delving into the neglected field of epigenetic regulation. His concluding remarks highlighted the planned WG1 positional paper publication as well as organization of a training school on systems biology.
*comment/suggestion by *Joe Chappell, US*: the target audience of the training school should include not only young, but also experienced researchers.

Polyphenols were the featured topic of the following presentation by *Stefan Martens, IT*. While introducing the recent progress in the elucidation and metabolic engineering of the pathways of interest, he invited the participants to consider the side reactions accompanying the formation of the valuable metabolites in question, and the remarkably poor understanding thereof, as exemplified by glucosylation and polymerisation processes. He further introduced a hypothesis of polyphenol metabolon formation and its implications for pathway regulation, subcellular organization and metabolite channelling. Moreover, he addressed the exploitation of microorganisms as expression

systems for flavonoids. The concluding remarks of the talk dealt with the possible alternative (non-GMO) modifications in plants, as exemplified by transient expression, up-regulation of endogenous transcription factors, efficient hybrid creation and elicitation.

Peter Facchini, CA introduced the recent advances in the understanding of the benzoisoquinoline alkaloid biosynthesis and its cellular localization, based on novel *in planta* technologies such as virus-induced gene silencing (VIGS) and LC-MS/MS shotgun proteomics (featured in detail in: Hagel, J.M. & Facchini, P.J., 2013, *Plant Cell Physiol.* doi:10.1093/pcp/pct020).

In his talk on biocatalysis and industrial approaches to salvaging valuable plant natural products, *Ludger Wessjohann, DE* drew the participants' attention to the economic aspects of the issue at hand, emphasizing the superiority of 'market pull' over 'technological push'. He further gave voice to the serious environmental and ecological implications of large-scale fermentation endeavours with plant-derived biocatalysts. In conclusion, he postulated taking full advantage of classical chemical approaches before employing biocatalytic methods.

WG2 Molecular Tools:

North American experience in deep sequencing and metabolomics

In his second address, *Peter Facchini, CA* reported on the *PhytoMetaSyn Project* (http://www.phytometasyn.ca/) and its main objectives: the establishment of a genomics pipeline integrating massively parallel DNA sequencing, targeted metabolomics, advanced bioinformatics and 'plug-and-play' functional genomics in yeast to efficiently identify, characterize and catalogue an expanding collection of biosynthetic genes responsible for the immense diversity of plant metabolism; the development of a framework for the commercial production of valuable PNPs in microbial systems; and the demonstration of the feasibility of synthetic biology as a platform for the production of six prototype plant natural products (nootkatone, abietic acid, betulinic acid, morphine, strictosidine and xanthohumol) in engineered yeast. While discussing the challenges encountered in course of the *Project*, such as the selection of appropriate promoters and transcription terminators to promote efficient recombinant gene expression for optimized pathway flux, he highlighted its achievements: the establishment of a genomics and metabolomics database of 75 plant species – rationally selected producers of valuable natural products, and a parts catalogue for BIA biosynthetic enzymes spanning 20 species.

Joe Chappell, US introduced the outcome of a parallel initiative undertaken by the *Medicinal Plant Consortium* in the United States (http://medicinalplantgenomics.msu.edu/). He enumerated the objectives of the *Consortium* and its free-access resource:

- obtain well-characterized and reproducible samples of plant materials for the 14 taxonomically diverse medicinal plant species; for each species, up to 20 tissue samples will be selected that are anticipated to have significant variation in concentrations of medicinal compounds: 10 core tissue samples and up to 10 additional samples selected by the relevant species experts; each sample will be extracted for RNA and metabolites and aliquots provided to the metabolomics and transcriptomics units for analyses;
- perform quality control on samples using LC-MS to assess the levels of 5-10 known, well-characterized medicinal compounds in each plant species; these validated, chemically diverse samples will then be used for whole transcriptome sequencing, gene expression profiling and quantitative metabolite profiling analyses;
- obtain 600-800 Mb of transcriptome sequence per species using next generation sequencing of a normalized library made from pooled mRNA from 5 diverse tissues (e.g., roots, stems, flowers, young leaf buds and callus tissue); generate a virtual transcriptome for each species; annotate the assembled transcriptome for putative gene function using bioinformatic approaches including sequence similarity, motif/domain searches and subcellular localization predictions;
- employ Illumina RNA-Seq whole transcriptome sequencing to generate deep expression profiles in up to 20 chemically diverse tissues for each plant species; map expression data to assembled virtual transcriptome of each species; use data to further improve the transcriptome assembly;
- perform quantitative metabolite profiling of the samples to quantify the relative levels of known medicinal compounds and potential metabolic intermediates in each species;
- deposit all datasets into the relevant publicly accessible databases and make raw and processed datasets available through the project website; this site will provide a user-friendly data interface and will incorporate custom tools for the community to download, access and compare the sequences, annotations, transcript expression and metabolite data sets; the data and website will enable a key, previously inaccessible link between the genome and metabolome of medicinal plants, and provide novel information to the community about the genes and markers of medicinal compound synthesis.

At present, the metabolomics resources from *Atropa belladonna* and *Digitalis purpurea* are accessible, while the metabolite data from the remaining 12 medicinal plant species covered by the project will be made available soon.

WG2 Molecular Tools:

PNP bottlenecks

Major bottlenecks encountered in plant natural product research and possible solutions to the issue were discussed by *Sarah O'Connor, UK*. While gene clusters are considered a hallmark feature of microorganisms, she drew the participants' attention to the hypothesis of clustering of biosynthetic pathway genes in plants, as exemplified by the recent identification of a 10-gene cluster for synthesis of the anticancer alkaloid, noscapine in opium poppy (Winzer *et al*., 2012, *Science* 336: 1704). Furthermore, she emphasised the potential of innovative technologies (VIGS) and the need for transcriptome analysis of alternatively bred cultivars (high- vs. low-producing) for better understanding of gene expression levels.

Ian Graham, UK presented the goals and results of his work on molecular breeding for improvement of plant natural products. Featured topics included the development of novel oil (*Jatropha curcas*) and medicinal (*Artemisia annua* and *Papaver somniferum*) crops. *Ian* emphasized the successful establishment of improved, artemisinin-rich varieties of sweet wormwood, genetic mapping of the plant as well as the combined efforts to deliver improved hybrid seed into the developing world supply chain (http://www.york.ac.uk/org/cnap/artemisiaproject/index.htm).

Stefan Schillberg, DE, a representative of the *Fraunhofer Corporation*, presented recent technological advances in large-scale, industrial plant cultivation, propagation and harvesting, highlighting a newly designed *Fraunhofer* facility, a so-called 'vertical farm'. He further addressed innovative approaches, such as production of valuable compounds in cell-free systems. In conclusion, he drew the participants' attention to the development and testing of an antibody against HIV and the *Corporation's* objective that it be applied in clinical studies (http://www.fraunhofer.de/en/press/research-news/2011/may/medicines-from-plants.html).

WG2 Molecular Tools:

Synthetic biology/metabolic engineering tools

In her second address, *Sarah O'Connor, UK* discussed the challenges of plant natural product pathway reconstitution in microbes. She presented examples of successful trials: flavonoid biosynthesis in yeast and *E. coli*, production of artemisinic acid in yeast for further semi-synthesis into antimalarial artemisinin, partial expression of morphine biosynthetic genes in yeast and *E. coli* and fine-tuned and optimized bacterial expression levels of genes encoding early enzymatic steps of taxol biosynthesis. However, the question of the preferable PNP production platform being of plant or microbial origin remained open.

In reporting on the characteristics and amendments to the *Golden Braid Modular DNA Construction System*, *Diego Orzaez, ES* presented the expanded features of the *GB 2.0* software package, assisting users in the design and virtual assembly of new genetic parts and devices conforming to the *GB* standard (http://www.ibmcp.upv.es/FGB/GB/GB2.0_Users%20manual70.htm).

In conclusion, he encouraged the participants to take full advantage of the innovative universal system, to help establish *Golden Braid* as the versatile standard for future modular cloning endeavours.

In the final talk of the day, *Sylvestre Marillonet, DE* presented an alternative, yet *GB*-compatible, Modular Cloning System (*MoClo*) for further universal standardization of DNA assembly trials. He especially highlighted the expanded version of the system, *MoClo Pro*, facilitating efficient gene expression in *Prokaryotes*, as exemplified by successful attempts of carotenoid and violacein production in microbial hosts. He emphasized the versatility of the system and expressed his hope that, along *GB 2.0*, *MoClo* be accepted and universally applied by the PNP research community.

Group Discussions

Following the presentation sessions, the participants discussed their expectations as to the immediate goals of the *PlantEngine* initiative in four groups.

The discussion coordinators were:

1) *Antonella Leone, IT*,
2) *Mariana Sottomayor, PT*,
3) *Paul Fraser, UK*,
4) *Heribert Warzecha, DE*.

Friday, March 1

After a short welcoming address to the second day of proceedings, the MC Chair, *Heribert Warzecha, DE* asked the group coordinators to shortly summarize the discussions of the previous evening.

Ad. 1) main concerns and objectives:

- promote the broad application of the standardized *Golden Gate/Golden Braid* technologies within the scientific community, with a strong emphasis on the aspects of gene regulation (extensive testing and optimization of alternative promoter, terminator and RBS sequences);

- concentrate efforts on verification of enzyme function, including pleiotropic effect prediction; establishing a repository of rate-limiting precursors and intermediates as well as an optimized bioinformatics tool-set for enzyme structure-function computing;
- focus on cellular logistics: aspects of biosynthetic pathway expression, localization and transport, in microbial vs. plant systems.

Ad. 2) main concerns and objectives:

- create a web-based tool to store and make accessible -omics data generated for selected species producing high value PNPs (next-generation sequencing + metabolomics);
- establish a genome sequencing project of target medicinal species to look for gene clustering in secondary metabolism;
- invest in one or two projects to reach the market level and post as success stories (like, e.g. golden rice or blue carnation); *systems biology* as a viral catchword;
- create a repository of parts for plant metabolic engineering (*Golden Gate/Golden Braid Systems*); the problem of quality control of such a repository was raised, and it was suggested that instead of establishing an entirely new endeavour, the scientific community could make use of already existing structures, such as *Addgene*).

Ad. 3) main concerns and objectives:

- broadly utilise the new combinatorial cloning systems (*Golden Braid/MoClo*) within the community; issues raised were infrastructure support and IP on the repository parts;
- focus on fundamental biochemical gene validation & characterisation that underpin exploitation of valuable species;
- attract funding for community resources: databases, authentic standards and chemicals;
- re-address the exploitation of natural variation;
- allocate resources to elucidate new levels of genome regulation.

Ad. 4) main concerns and objectives:

- define 'flag-ship' projects within the *Action* to attract attention, support and funds; suggested research areas: molecular breeding of valuable, non-model species, biosynthetic pathway engineering;
- focus on the approaching mid-term review in Brussels; a back-to-back meeting with a coinciding EU-China scientific convention dealing with bio-economic and political issues was suggested, to take advantage of the extended audience of decision makers and industry representatives, and further lobby and advertise the cooperative effort of *PlantEngine*;
- key recommendation: open access facility for synthetic biology (*Golden Braid 2.0*);

- highlight the need to preserve European biodiversity and select priority species of interest for the establishment of Pan-European -omics database; focus especially on food- and high value product crops to address the existing economic crisis and thus influence the decision makers (*use European resources to address European problems*).

As a general consensus concerning the immediate *PlantEngine* objectives could be traced throughout all the group discussions, the following salient points were identified and further considered:

1) Computational PNP Research,
2) Biochemistry & Cellular Logistics,
3) Technologies,
4) Synthetic Biology.

Ad. 1) discussion chair, *Paul Fraser, UK*.

Main discussion points and recommendations:

- tools and strategies for better metabolite annotation and pathways elucidation in non-model spp;
- tools to use and validate the data;
- a set of standardised reference extraction, separation and MS protocols;
- an international aspect: broad global acquisition of knowledge and resources;
- separation of systems biology from pathway modelling; the need for quantitative data delivery;
- high-throughput functional genomics;
- lobby national funding agencies to start the process and tackle the aforementioned obstacles.

Ad. 2) discussion chair, *Maria Angeles Pedreño, ES*.

Discussion report:

We found that before giving specific recommendations, it is necessary to define at what level the future studies should be carried out: whole plant, organs, tissues, cells or subcellular level. There is also a need to define which secondary metabolites would be interesting to localize. The answer to these questions will determine the choice of the technique to be applied: tissue fractionation, protoplast/vacuole isolation, any other tool to isolate organelles, histo-, immuno- and cyto-chemical studies. The possibility of performing *in situ* metabolite localization using reporter aptamers or fluorescent probes was discussed, and it was recognized that there is a need for supporting and developing this technique.

However, the main problem to address within this research area is the vast number of different plant secondary metabolites, as stated by Wu and Chappell in *Curr. Opin. Biotech*, 2008: *There are approximately 300 000 documented species of higher plants on the planet with more than 200 000 individual chemical entities, compounds referred to as NPs (including primary and secondary metabolites)*. This fact is more evident in the case of alkaloids, since their biosynthesis is more complex through the involvement of multiple cellular compartments and tissue-specific transformations (as indicated by *Peter Facchini, CA*).

Therefore, it is necessary to define, what the nature of a given secondary metabolite of interest is, before establishing any recommendation on how to proceed. PNPs can be classified into large groups based on their biosynthetic origins, that is, in three main classes: isoprenoid-derived compounds, alkaloids and polyphenolics, although other compounds, like glucosinolates and cyanoglucosides, may be also considered as another important group.

Another important question to deal with is how intercellular and intracellular metabolite trafficking is conducted. We can try to elaborate a flow chart in each case (depending on the nature of a metabolite) to show how the metabolite is transported and what kind of transporter (specific or unspecific) is involved.

Finally, we also discussed the importance of using standardized methods for metabolite extraction, as it is impossible to have one optimized extraction method for all types of plant tissues. The nature of the solvent is the most important factor involved in an optimal quantitative extraction and its selection depends on the structure of plant tissue, the binding state, the type of extracted metabolites and on whether the objective is quantitative or qualitative, so that not only extraction but also identification and quantification could be considered.

In conclusion, we listed the recommendations for a more efficient production of valuable compounds, in relation to the cellular logistics:

- define the nature of each secondary metabolite of interest, in order to know how to proceed, as, in some cases, there is a lack of knowledge on where the metabolite is located or whether its biosynthesis involves different cellular compartments;
- define at what level (whole plant, organ, tissue, cell, etc.) the study is going to be carried out, before choosing the technique to determine the precise localization of the compound of interest;

- there is an urgent need to understand intracellular and intercellular metabolite trafficking in PNP pathways, which should involve:
  - ✓ optimization of analytical methodologies through:
    - organization of libraries of compounds,
    - standardization of methodologies and equipment for better comparison and sharing of data,
    - sharing of mass-spectrum data by encouraging researchers to upload their data in Massbank (include the link on the *PlantEngine* website);
  - ✓ intensification of research on metabolite transmembrane transporters;
  - ✓ promoting of and training in cell fractionation techniques and *in situ* metabolite localization techniques within the PNP research community (scinthenols(?), aptamer probes, MS imaging) – a need to interact with cell biologists.

Ad. 3) discussion chair, *Ian Graham,UK*.

Discussion report:

Recommendations for Future EU Focus for Development of Natural Products

Rapid Domestication through Molecular Breeding

A number of examples presented during the course of the meeting in Porto and in the recent literature demonstrated that the time is now right to seriously consider rapid domestication of plant species that produce high value chemicals.

It was recognised that the platform technologies and know-how exist among European scientists and companies to embark on ambitious programmes that will realise the potential of plants as green factories for high value products.

Development of new crops producing high value chemicals for Europe could have a major impact on economic growth and environmental sustainability.

In particular the following points were discussed:

- in the identification of target species for rapid domestication, there was general consensus that it would be preferable to exploit European Biodiversity if possible; it was agreed that plants producing high value natural products would need demonstrable pull from industry and/or supporting economic analysis to justify selection;
- it was recognised that the recent FP7 KBBE call on Plant High Value Products was focussed mainly on bio-discovery; this will lead to a number of projects being funded by the EC on high value products but these typically do not include a

facility for rapid improvement of the plants themselves; thus, if this topic is to be taken forward with EU funding, it will be important to ensure synergy and added benefit with existing and previously funded projects;

- it was recognised that Europe was well catered for in terms of access to genetic resources, primarily from European Botanic Gardens;
- chemotyping and genotyping platforms would be essential in initial characterisation of potential targets, as this would allow genetic distance and level of natural variation available for breeding to be defined;
- development of technologies to assist in generating additional genetic variation and new genetic resources as well as inter- and intra-specific crosses to enable outbreeding could be particularly valuable; rosemary and sage were considered as examples of plants that could benefit from these technologies;
- in addition to focussing on yield of natural products, it was agreed that improving other traits was also essential for rapid domestication; it was agreed that defining agronomically important traits for yield improvement would be an essential first step;
- this is already true for existing European crops such as lavender, which currently lacks good disease resistance, putting crop production at risk;
- it was agreed that it may be possible to develop generic tools that could be used to improve yield of various natural products in a specific organ, such as glandular trichomes or roots;
- epigenetics was recognised as a key area for further research into the regulation of natural product biosynthesis; understanding the role of epigenetics could lead to improved agronomic practices for crop based natural products production;
- *Lost Crops* – there was a general consensus at the end of the discussion that time was now right for Europe and the rest of the world to re-introduce *Lost Crops* that produce bio-renewables to replace petrochemically derived feed-stocks in what is a move back to sustainable green production of various commodities including flavours, fragrances, colourants etc.

Platform Plant Species for Specific Natural Products

In addition to discussing research and development leading to rapid domestication, the participants also considered the possibility of developing specific platforms for production of different classes of natural products. These applications would generally require GMO based approaches.

For example:

- ✓ *Nicotiana tobaccum* for diterpenoids,
- ✓ *Artemisia annua* for sesquiterpenes,
- ✓ *Centella asiatica* for triterpenoids,
- ✓ *Papaver somniferum* for benzylisoquinoline alkaloids (BIAs).

Ad. 3) discussion chair, *Heribert Warzecha, DE*.

Main discussion points and recommendations:

- recognize and acknowledge the *Golden Braid/MoClo* technology implementation as a main and tangible outcome of the *PlantEngine* initiative;
- identify the technical issues to be dealt with:
  - ✓ centralised repository of standardized *GB 2.0/MoClo* parts:
    - independent, or
    - in cooperation with existing non-profit organizations (e.g. *DSMZ*, *Addgene* and, most importantly, *BioBrick Foundation*);
  - ✓ IP issues & quality control standards to be applied:
    - the deposited parts should be sequenced,
    - all other aspects should be validated by the user,
    - formulate an appropriate disclaimer (*BioBrick Foundation* agreement as a blueprint, *Heribert Warzecha, DE*);
  - ✓ future distribution:
    - a technician position should be created to facilitate the distribution of *GB 2.0 starter kits* and sequencing of newly deposited parts;
  - ✓ immediate distribution of starter kits for interested participants:
    - define cost projections for the starter kit delivery handling fee (*Diego Orzaez, ES*);
  - ✓ promotion:
    - cross-reference links on both *PlantEngine* & *IBMCP-FGB* websites,
    - *Golden Braid* training school (September 2013, Valencia, Spain): *e-learning* – online streaming of the lectures and practical courses;
  - ✓ appropriate acknowledgement of the designers' effort:
    - citation of relevant papers,
    - scientific incentive within the broad research community.