Engineering Marchantia polymorpha chloroplasts for the production of high-value specialized terpenes

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The Idea

Land plants produce a huge variety of chemicals. Some of these are primary metabolites essential for basic metabolic processes involved in plant growth, development and reproduction. The other non-essential chemicals are called secondary metabolites, specialized metabolites or natural products, and have important ecological functions primarily in plant defence against herbivores, pests and pathogens. These natural products are not only useful to the plants that produce them, but also have powerful physiological effects in humans [1]. Plant parts and extracts have been traditionally used in different cultures for the treatment of a wide range of ailments. In modern medicine, plant natural products, their derivatives and analogues represent more than half of all clinically used therapeutics. Plant natural products are also consumed by humans in everyday foods as flavours and fragrances. This makes land plants a very rich source of natural products with diverse and useful properties for humankind. However, the use of many natural products is restricted by their limited accumulation in the plant, slow growth rate of the plant and varying levels of compound accumulation that are susceptible to geographical and environmental conditions. In addition, extraction of natural products most often involves the use of destructive methods on the source and can be uneconomical. Hence, engineering heterologous hosts that are easy to manipulate, maintain and cultivate for the production of natural products with economic value is of great interest [2].

The terpenes represent a large class of structurally diverse plant natural products. They are derived from the repetitive fusion of isoprene (C5) units, and are classified as hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), tri- (C30), tetra- (C40), or poly- (C>40) terpenes based on the number of units they contain. Because of their structural variety, the terpenes display a wide spectrum of biological activities [3]. For instance, the monoterpene geraniol, the sesquiterpene amorpha-4,11-diene and the triterpene β-amyrin are precursors of the potent anti-cancer drug vincristine, the anti-malarial drug artemisinin, and bioactive triterpenoid saponins, respectively. In plant cells terpene biosynthesis is highly compartmentalized [2]. The precursors for hemi-, mono-, di-, tetra- and polyterpene synthesis are generated in the chloroplast, while the sesqui- and triterpene precursors are synthesized in the cytoplasm. Ongoing research in the Osbourn lab (JIC) supports the notion that triterpene production in Nicotiana benthamiana can be improved by directing its biosynthesis to non-native cellular compartments, like the chloroplast.

The thalloid liverwort Marchantia polymorpha has recently received marked attention as a basal multicellular plant chassis. On account of its simple propagation, high regenerative capacity, and established tools for nuclear and chloroplast transformation, M. polymorpha shows great promise as a heterologous host for metabolic engineering. Liverworts naturally synthesize enzymes and secondary metabolites of commercial relevance, including antifungal [4], antimicrobial [5] and anticancer [6] agents. In addition, suspension cultures of liverworts have been used in the biotransformation of organic substrates and photoautotrophic growth conditions have been established allowing their large-scale cultivation in bioreactors [7]. In M. polymorpha, although the

production of specialized terpenes has not been reported, several isoprenoid biosynthesis enzymes catalyzing the synthesis of terpene precursors have been identified and shown to have similar subcellular compartmentalization as land plants [8]. Through this project, we aim to pioneer metabolic engineering in chloroplasts of M. polymorpha, and assess the potential of exploiting them as a production chassis for high-value specialized terpenes. As a proof-of-concept the heterologous production of geraniol, amorpha-4,11-diene and β-amyrin in chloroplasts will be evaluated.

1. Moses, T., K.K. Papadopoulou, and A. Osbourn, Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. Critical Reviews in Biochemistry and Molecular Biology, 2014. 49(6): p. 439-462.
2. Moses, T., et al., Bioengineering of plant terpenoids: from metabolic engineering of plants to synthetic biology in vivo and in vitro. The New Phytologist, 2013. 200(1): p. 27-43.
3. Croteau, R., T.M. Kutchan, and N.G. Lewis, Natural Products (Secondary Metabolites), in Biochemistry & Molecular Biology of Plants, B. Buchanan, W. Gruissem, and R. Jones, Editors. 2000, American Society of Plant Biologists. p. 1250-1318.
4. Niu, C., J.B. Qu, and H.X. Lou, Antifungal bis[bibenzyls] from the Chinese liverwort Marchantia polymorpha L. Chemistry & Biodiversity, 2006. 3(1): p. 34-40.
5. Mewari, N. and P. Kumar, Antimicrobial Activity of Extracts of Marchantia polymorpha. Pharmaceutical Biology, 2008. 46(10-11): p. 819-822.
6. Huang, W.J., et al., Marchantin A, a cyclic bis(bibenzyl ether), isolated from the liverwort Marchantia emarginata subsp. tosana induces apoptosis in human MCF-7 breast cancer cells. Cancer Letters, 2010. 291(1): p. 108-119.
7. Chiou, S.Y., W.W. Su, and Y.C. Su, Optimizing production of polyunsaturated fatty acids in Marchantia polymorpha cell suspension culture. Journal of Biotechnology, 2001. 85(3): p. 247-257.
8. Suire, C., et al., Cellular localization of isoprenoid biosynthetic enzymes in Marchantia polymorpha. Uncovering a new role of oil bodies. Plant Physiology, 2000. 124(3): p. 971-978.

Who We Are

1. Aymeric Leveau
Post Doctoral Scientist, Department of Metabolic Biology, John Innes Centre.
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I am a synthetic biologist currently engineering wheat for take-all resistance. My work focuses on transferring genes responsible for the synthesis of Avenacin A-1, a triterpene saponin naturally produced by oats that confers extreme resistance against the soil born root pathogen Gaeumannomyces graminis, into wheat. This involves identifying and characterising the missing genes of the avenacin biosynthetic pathway and developing molecular cloning tools that will allow the transfer of the 11 genes into wheat. I have research experience in multi-gene construct building (primarily using GoldenGate assembly) and heterologous system gene expression.

2. Tessa Moses
Post Doctoral Scientist, Department of Metabolic Biology, John Innes Centre.
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I am a synthetic biologist currently developing an in vivo biosensor in the yeast Saccharomyces cerevisiae to facilitate in-cell detection of heterologously produced bioactive compounds with anti-inflammatory activity. I have research experience in metabolically engineering yeast for the production of natural and unnatural terpenoids (triterpenoids in particular), and in metabolite analysis using chromatography techniques (GC-MS in particular).

3. Christian R. Boehm

Graduate Student, Department of Plant Sciences, University of Cambridge.
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I am a synthetic biologist genetically engineering M. polymorpha chloroplasts. My research experience relevant to this proposal embraces multi-gene construct building, metabolic engineering in E. coli (aimed at fatty acid derivatives in particular), propagation and transformation (nuclear and plastid) of M. polymorpha, and in vivo monitoring of plastid gene expression using confocal microscopy and image analysis techniques.

Implementation

The funding will be split among the following work packages:

1. Design and assembly of terpene constructs

Due to the restricted availability of chloroplast gene promoters and in order to keep the inserts as small as possible, three independent operon-like synthetic constructs will be designed to achieve de novo synthesis of mono-, sesqui- and triterpenes in M. polymorpha chloroplasts. The GoldenGate Modular Cloning (MoClo) system will be used to assemble the multi-gene expression cassettes using modular components shared among members of the OpenPlant community. The coding sequence of the full-length genes to be expressed will be synthesized to allow M. polymorpha chloroplastic codon optimisation and GoldenGate domestication. We will build three binary vectors containing the monoterpene (Plant Kanamycin resistance – p35S::CPMV 5'UTR ::GES::P2A::GPPS::P2A::Turq2mp:: CPMV 3'UTR::t35S – p35S::p19::t35S), sesquiterpene (Plant Kanamycin resistance – p35S::CPMV 5'UTR ::ADS::P2A::FPPS::P2A::Turq2mp:: CPMV 3'UTR::t35S – p35S::p19::t35S), or triterpene (Plant Kanamycin resistance – p35S::CPMV 5'UTR ::BAS::P2A::SQE::P2A::SQS::T2A::FPPS::T2A::Turq2mp:: CPMV 3'UTR::t35S – p35S::p19::t35S) expression cassette in the Level 2 GoldenGate vector pAGM4723 [9]. The genes of interest (red) are separated by the porcine teschovirus-1 2A (P2A) or the Thosea asigna virus 2A (T2A) sequences (bold) that encode self-cleaving 22 amino acid peptides [10]. The 2A sequences cause the ribosome to skip the synthesis of the terminal glycyl-prolyl peptide bond at the C-terminus of the peptide, leading to the cleavage between a 2A peptide and its immediate downstream protein. As a result, the cleaved-off downstream enzyme has a proline at its N-terminus and the upstream protein harbours a 21 amino-acid peptide at its C-terminus.

The assembly and functioning of the expression cassettes will be first validated in N. benthamiana leaves, a day after Agrobacterium-mediated infiltration, by evaluating expression of the turq2mp reporter gene (cyan) using confocal microscopy. This gene encodes the mTurquoise2 fluorescent protein, which exhibits the highest reported quantum yield among green fluorescent protein (GFP) derivatives [11]. To date, this codon-optimized gene is the only GFP variant successfully expressed in chloroplasts of M. polymorpha [12]. Following this validation, the terpene expression cassettes (underlined) will be PCR amplified and cloned into the M. polymorpha pCS CL vector [12] using Gibson assembly to generate mono-, sesqui-, or triterpene cassettes under the control of the tobacco psbA promoter. The expression cassettes containing the turq2mp reporter gene will be flanked by chloroplast homologous regions trnG and trnfM at the 5'- and 3'-termini, respectively, and in addition carry the aadA cassette conferring spectinomycin resistance. The mTurquoise2 fluorescent protein will be used to visually screen efficient transcription and translation from the polycistronic mRNA.

1. Biolistic transformation of M. polymorpha chloroplasts

M. polymorpha chloroplasts will be transformed using a previously reported biolistic approach [13]. The relevant workflow is well established in the Haseloff lab, and has been utilized for the generation of several M. polymorpha transplastomic lines within the past year. Four weeks after particle bombardment of day 5-7 sporelings, regenerating plantlets will be screened for

transplastomic events by monitoring the cyan fluorescence from the turq2mp reporter gene. Plantlets with plastid-localized cyan fluorescence will be subjected to several rounds of propagation under spectinomycin selection to establish homoplasmy over a period of 3-4 months. Homoplasmy will be confirmed by a PCR-based assay, prior to metabolite analysis of the transplastomic lines.

1. Analysis of M. polymorpha for the production of terpenes

Organic metabolite extracts from M. polymorpha transformants will be analyzed using gas chromatography – mass spectrometry (GC-MS) for the production of geraniol, amorhpha-4-11-diene and β-amyrin. The GC-MS facility is housed in the Department of Metabolic Biology (JIC) and is accessible to members at a fee (see budget section). Well established protocols for metabolite extraction from plants and GC-MS analysis, along with commercial standards for the terpenes (geraniol, amorhpha-4-11-diene, β-amyrin) and the precursors (squalene, 2,3-oxidosqualene) are available in the Osbourn lab (JIC). The metabolite profiles of heterologous terpene-expressing M. polymorpha transformants will be compared to a control M. polymorpha transformant to identify metabolite peaks unique to the transformants, and their identities will be confirmed against authentic standards.

1. Engler, C., et al., A golden gate modular cloning toolbox for plants. ACS Synthetic Biology, 2014. 3(11): p. 839-843.
2. Kim, J.H., et al., High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One, 2011. 6(4): p. e18556.
3. Goedhart, J., et al., Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. Nature Communications, 2012. 3(751).
4. Boehm, C.R., et al., A fluorescent reporter gene for stable chloroplast transformation in Marchantia polymorpha. in preparation.
5. Chiyoda, S., et al., Simple and efficient plastid transformation system for the liverwort Marchantia polymorpha L. suspension-culture cells. Transgenic Research, 2007. 16(1): p. 41-49.

Benefits and outcomes

The proposed interdisciplinary project brings together terpene engineering expertise in the Osbourn lab (JIC) and the M. polymorpha chloroplast transformation platform in the Haseloff lab (University of Cambridge), thereby promoting knowledge exchange between Norwich and Cambridge. The success of this project can generate publishable results in a short space of time and will be pioneering metabolic engineering in M. polymorpha chloroplasts. The promising outcome of this project can also extend to follow-up work on further modifying the produced terpene backbones with known biosynthetic enzymes, or engineering M. polymorpha chloroplasts for other terpenes like the diterpene taxa-4,11-diene, which is the precursor of the anti-cancer drug taxol.

Budget

Technique	Cost/sample	No. of samples	Total £
DNA synthesis for coding genes	£0.09/bp	24,147bp	2173.23
Assembly of terpene constructs	£17.50	4	70
M. polymorpha chloroplast transformation	£1.90/shot	240 shots (4 repeats x 3 constructs x 10 plates x 2 shots)	456

GC-MS	£19	17 (3 biological repeats x 4 constructs + 5 standards)	323
Total			3022.23