OpenPlant Fund Application Form

30th June 2017

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Title of Project

Engineering of *Chlamydomonas reinhardtii* to produce betalain pigments and the use of riboswitches to direct metabolic flux.

Primary contact for the team

Alfonso Timoneda Monfort
Department of Plant Sciences, University of Cambridge
at656@cam.ac.uk

Sponsor for the research and cost centre

Dr Samuel Brockington
Lecturer in Evolution and Diversity & Curator of the Cambridge University Botanic Gardens
Department of Plant Sciences, University of Cambridge
sb771@cam.ac.uk

I confirm that I have the full support of the sponsor listed above and that they can be added to the OpenPlant Fund mailing list to receive project updates (to which they can unsubscribe at any time).

The Team

Alfonso Timoneda
MPhil student and Research Laboratory Technician
Department of Plant Sciences, University of Cambridge
at656@cam.ac.uk

"I am currently an MPhil student in Dr Sam Brockington's group where I am focused on understanding the betalain biosynthetic pathway. My main research interests reside in the field of metabolic engineering and the transference of plant metabolic pathways to industrially scalable microorganisms in order to produce high value compounds. Betalains present many beneficial features to become target molecules for metabolic engineering. My previous knowledge on the pigmentation pathway and the expertise I recently developed on the use of the MoClo Golden Gate technology will contribute to the success of the proposed project.

Dr Payam Mehrshahi
Postdoctoral Scientist
Department of Plant Sciences, University of Cambridge
pm579@cam.ac.uk

"My research interests concern engineering of photosynthetic organisms to produce high value compounds. To achieve this, I am applying synthetic biology principles in Chlamydomonas reinhardtii, *to develop and test genetic circuits that allow fine-tuning of target metabolic pathways in this organism. The molecular and cell biology techniques that I have helped to develop, along with my experience in engineering of regulatory elements in* Chlamydomonas *will directly contribute to the success of this proposal.*

Summary

Betalain pigments are plant metabolites showing a wide variety of potential applications in the pharmaceutical, agricultural and food industry. Betalains are currently used as food and cosmetic colorants [1], and can act as potent antioxidant [2], anti-cancer [3, 4], anti-lipidemic [5, 6] and anti-microbial molecules [7]. Certain intermediates of the betalain metabolic pathway also have pharmacological properties e.g. L-DOPA is used for the treatment of Parkinson's disease [8]. Given these benefits, it is important to further understand the biosynthetic processes leading to betalain production and accumulation in plants and how this knowledge can be used to engineer new industrial production hosts using model organisms like microalgae.

The betalain pathway consists of three main enzymatic steps starting with the molecule tyrosine (Fig 1) [9]. The first step of the pathway displays an interesting enzymatic redundancy, where at least three cytochrome P450 proteins (CYP76AD1, 5 and 6) are able to perform the conversion of tyrosine to L-DOPA [10, 11]. CYP76AD1 can also catalyse the second reaction of the pathway, from L-DOPA to cyclo-DOPA, necessary for production of red coloration [12]. One molecule of cyclo-DOPA can then spontaneously condense with a molecule of betalamic acid, product of the DODA gene in the third step of the pathway [13], to give the visible red pigments, betacyanins. Alternatively, betalamic acid can also condense with amino groups to form the fluorescent yellow pigments, betaxanthins.

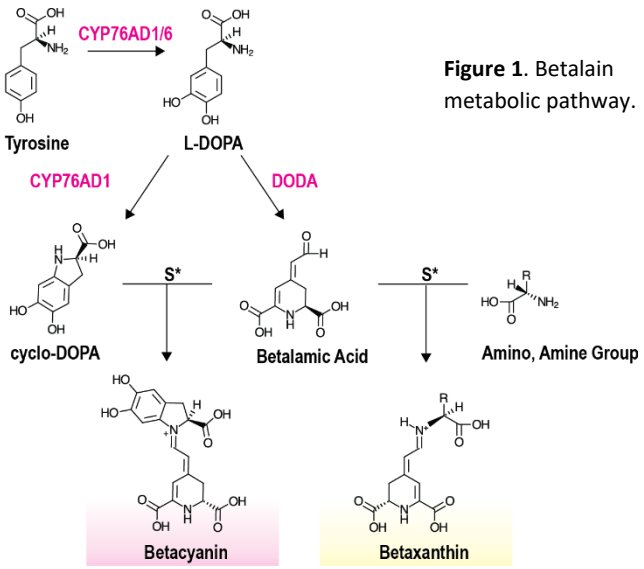

Figure 1. Betalain metabolic pathway.

Different CYP76AD enzymes are important for controlling the types of betalains produced and thus the resulting colour balance from pink to yellow via intermediate hues. Transient transformation of *Nicotiana benthamiana* with CYP76AD1 and the rest of the betalain biosynthetic machinery results predominantly in betacyanin production (red colour), whereas when CYP76AD6 is substituted for CYP76AD1, plants only produce betaxanthins (yellow colour) [10]. Orange colours in betalain-pigmented species have been shown to result from the presence of both betacyanins and betaxanthins [14]. Silencing of CYP76AD1 expression in beet has been shown to decrease betacyanin content and increase betaxanthins [12], indicating that modulating CYP76AD1 expression should influence the proportions of these two types of betalain pigments. Altogether, these findings suggest CYP76AD enzymes could have an important regulatory role in determining pigment composition and final coloration. The simplicity of the betalain pathway, the redundancy observed in the first step of betalain biosynthesis and the fact that betalamic acid acts as a shared chromophore for the two final pigment types, makes the betalain pathway an ideal system for visual characterisation of enzymes and their significance for metabolic flux towards either branches of this pathway.

We therefore propose to heterologously express the betalain biosynthetic pathway in *Chlamydomonas reinhardtii* with metabolic flux artificially regulated towards betacyanin or betaxanthin production. This regulation is possible by the use of characterised riboswitches that respond to exogenous supplementation of culture media with thiamine and hydroxyethyl thiazole (HET). Such riboswitch-driven regulation of the branch point enzymes will allow the ratio of cyclo-DOPA to betalamic acid to be "controlled" and thereby selectively shift the overall coloration produced towards the red or the yellow spectra respectively. The ultimate aim of this project is to engineer a strain of *Chlamydomonas reinhardtii* that not only will be able to produce betalain pigments but also will be susceptible to external fine tuning towards a desired color mix resulting in particular hues.

Proposal

To produce *Chlamydomonas reinhardtii* strains that are able to produce betalains in a regulated manner, the following milestones will have to be addressed.

(1) Codon optimisation and gene synthesis

We will design and synthesise the sequences of four betalain biosynthetic genes (CYP76AD1, CYP76AD6, DODA and cDOPA5GT) previously optimised to reflect codon usage in the nuclear genome of *Chlamydomonas reinhardtii.* The enzyme cyclo-DOPA 5-O-glucosyltransferase (cDOPA5GT) has been shown to be necessary in many cases for stabilisation of pigments (unpublished data).

(2) Vector design and construction

Using the MoClo Golden Gate technology we will design and assemble multigene cassettes that will be used for transformation of the *Chlamydomonas* nuclear genome. Genes will be placed under the control of the algal constitutive promoter HSP70:RBCS2 and carbonic anhydrase 1 (CA1) terminator. Riboswitch parts (Thi4 and Thi4mutEpa50 riboswitch platforms) will be provided by Dr Payam Mehrshahi and included before the start codon of both of the integrated cytochrome P450s. CYP76AD1 will be under the control of the Thi4 riboswitch, which responds to thiamine and HET ligands, while CYP76AD6 will be placed under the Thi4mutEpa50 riboswitch, a mutation of Thi4 that only ligates to thiamine. Level 2 vectors will be assembled with a paromomycin resistence cassette (See Table 1 for complete list of vectors).

(3) Transformation of *Chlamydomonas*

Chlamydomonas reinhardtii strain uvm4 will be transformed by electroporation with the developed vector. Transformants will be selected by growth in TAP media under paromomycin selection. Colour should be produced by successful transformants, therefore visual screening could facilitate the process.

(4) Strain characterization

Gene presence and expression levels will be assessed by PCR and qPCR. Betalain production and composition in transgenic *Chlamydomonas* lines will be analysed using a spectrophotometer by measuring absorbance of betacyanins and betaxanthins at 540nm and 475nm respectively.

(5) Riboswitch activation and tuning

Riboswitches are regulatory segments of the messenger RNA that bind small molecules resulting in a change in the protein translation, normally an inhibition. They are capable of directly sensing small metabolite concentrations, and so the level of inhibition can be precisely modulated [15]. In absence of ligands in the growth media, the betalain pathway will be fully functional. By adding the ligand thiamine, we will inactivate both the first and second steps of the pathway, therefore inhibiting all pigment production. When adding HET to the growth media instead, we will be able to inactivate only the CYP76AD1 driven step of the pathway that is responsible for producing cyclo-DOPA and ultimately betacyanins, the red pigments. A decrease in the cyclo-DOPA production would translate into more betalamic acid molecules available to react with amino groups and consequently a higher production of betaxanthins, the yellow pigments. Because riboswitches are sensitive to ligand concentration, the degree of inhibition will directly depend on the amount of ligand added to the media, and therefore a whole gradient of coloration ranging from red to yellow could be achieved with the same *Chlamydomonas* strain.

Table 1. List of Golden Gate vectors to construct.

Level 1

Promoter	Riboswitch	CDS	Terminator
HSP70:RBCS2	-	DODAα1	CA1
HSP70:RBCS2	-	cDOPA5GT	CA1
HSP70:RBCS2	Thi4	CYP76AD6	CA1
HSP70:RBCS2	Thi4mutEpa50	CYP76AD1	CA1

Level 2

Position 1	Position 2	Position 3	Position 4	Position 5
Paromomycin	CYP76AD6	CYP76AD1	DODAα1	cDOPA5GT

Table 2. Gant Chart and proposed workflow.

Month	September				October				November				December				January				February			
Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Milestone (1)																								
Milestone (2)																								
Milestone (3)																								
Milestone (4)																								
Milestone (5)																								

Benefits and outcomes

Metabolic routes are normally better understood at a genetic level than they are at a regulatory level, where the knowledge of the relative contributions of enzyme and metabolite concentrations in controlling flux is limited. Developing standardized tools that allow us to control the direction and intensity of the metabolite flux is of a great interest for the field of metabolic engineering. The generation of pathways where enzyme activity could be tuned to optimise sequential reaction efficiency could translate into an important improvement of final product yield and a minimisation of intermediate metabolite toxicity. At the same time, it would offer the possibility of obtaining alternative final products.

The simplicity of the betalain pathway, the redundancy observed in the first step of betalain biosynthesis and the fact that betalamic acid acts as a shared chromophore for the two final pigment types, makes the betalain pathway an ideal system for visual characterisation of enzymes and their significance for metabolic flux towards either branches of this pathway. Moreover, betalains are molecules with recognised value in the pharmacological, food and cosmetic sectors as well as interesting for the development of tools in synthetic biology due to their spectroscopic characteristics. Betaxanthins are naturally fluorescent molecules [16], giving the project the potential to generate new screening tools as well as fluorescent markers to test gene circuits and regulatory elements in the fields of synthetic and cell biology.

The success of the project would generate publishable results within a relatively short amount of time as well as help develop open and standardized resources for the scientific and metabolic engineering community.

Budget

	Elements		Cost / Element	Cost
Gene synthesis	CYP76AD1	(1494 bp)	£0.19 / bp	£283.86
	CYP76AD6	(1523 bp)		£289.37
	DODAα1	(828 bp)		£157.32
	cDOPA5GT	(1503 bp)		£285.57
Sequencing	Source BioScience (100x)		£4.95 / rxn	£495
Chemicals	Thiamine			£18.5
	Hydroxyethyl thiazole (HET)			£20
Consumables	PHIRE Plant Direct PCR Kit (2x)		£166,43 / 500rxn	£332,86
	RNEasy Plant Mini Kit (2x)		£ 209,85 / 50 rxn	£419.7
	SuperScript III Full Kit		£516.8 / 50 rxn	£516.8
	PHUSION DNA polymerase		£59.95 / 100U	£59.95
	T4 DNA ligase		£179.95 /100,000U	£179.95
	BsaI (Type II RE)		£44 / 1,000U	£44
	Bpi (Type II RE)		£19.13 / 200U	£19.13
	NucleoSpin Plasmid prep kit		£149.95 / 250rxn	£149.95
	PureLink Gel extraction kit		£23.29 / 50rxn	£23.29
	Electroporation cassettes		£115.92 / 50cuv	£115.92
	Spectrophotometer cuvettes (5x)		£3.54 /100cuv	£17.7
	96-well plate for PCR (5x)		£14.95 / 10pl	£74.75
	96-well plate for algal culture		£37.37 / 50pl	£37.37
Approximate TOTAL				**£3540.99**

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