OpenPlant Fund Report

1st March 2018

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Title of Project

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

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Summary

Betalain pigments are plant metabolites showing a wide variety of potential applications in the pharmaceutical, agricultural and food industry. Given the potential shown, 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. We proposed to heterologously express the betalain biosynthetic pathway in *Chlamydomonas reinhardtii* with metabolic flux artificially regulated towards betacyanin or betaxanthin production by the use of riboswitches that respond to exogenous supplementation of culture media with thiamine and hydroxyethyl thiazole (HET). Such riboswitch-driven regulation of the branch point enzymes would 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. We have synthesised the sequences of five betalain biosynthetic genes (CYP76AD1, CYP76AD6, DODAα, and cDOPA5GT) previously domesticated and optimised to reflect codon usage in the nuclear genome of *C. reinhardtii*. We also isolated two recently published constitutive promoter and terminator combinations that constitute new tools for gene expression in *C. reinhardtii* (RPL23, FDX1). Five different multigene vectors were successfully assembled via MoClo Golden Gate cloning and transformed in the nuclear genome of *C. reinhardtii*. Selection and confirmation of expressing clones will be done in the near future and further analysis will determine riboswitch ability to modulate the betalain pathway.

Report and outcomes

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

(1) Codon optimisation and gene synthesis

We synthesised the sequences of five betalain biosynthetic genes (CYP76AD1, CYP76AD6, DODAα, and cDOPA5GT) previously optimised to reflect codon usage in the nuclear genome of *C. reinhardtii. Beta vulgaris* transcript sequences were used as templates for the codon optimisation for all genes, except for cDOPA5GT which was retrieved from the *Mirabilis jalapa* transcriptome. Domestication was done against *BpiI*, *BsaI* and *SapI* restriction sites to ensure compatibility with MoClo and Loop Golden Gate assembly protocols. Synthesis, domestication and codon optimisation were performed by the *GeneArt* gene synthesis service provided by Thermo Fisher Scientific. In all cases, genes were synthesised with flanking regions containing compatible MoClo Golden Gate overhangs and *BsaI* restriction sites, and entry plasmids were generated including a kanamycin resistance gene. Plasmid design was performed in order to be able to be used directly as MoClo level 0 vectors.

See Table 1 for complete list of generated vectors.

(2) Cloning of RPL23 and FDX1 promoter and terminator pairs

Shortly after starting the OpenPlant project, a report was published identifying new genic flanking sequences for improved transgenic expression in *C. reinhardtii* [1]. Initially, our list of available constitutive promoters for *Chlamydomonas* consisted of the well characterised HSP70:RBCS2 and PSAD. The amount of genes we were considering for our final multigene constructs and the low number of promoter/terminator pairs available translated into a considerable risk of silencing associated with the use of repetitive elements. We therefore aimed to isolate the recently characterised promoter and terminator sequences for the RPL23, RPL35 and FDX1 genes from *C. reinhardtii* genomic DNA. Primer design included the overhangs and *BpiI* restriction sites required for cloning into MoClo level 0 acceptors. Promoters were amplified using two different primer pairs, generating amplicons with either TACT or AATG 3' overhangs, for their use as promoter only or promoter plus 5'UTR parts respectively (Figure 1). Amplification was successful for promoter/terminator pairs of RPL23 and FDX1, and was followed by gel extraction and cloning to corresponding level 0 acceptors. This was enough to complete our multigene construct design and further attempts to isolate RPL35 flanking sites were not performed, but can be done in the future.

See Table 1 for complete list of generated vectors.

(3) Multigene vector construction

Using the MoClo Golden Gate technology we assembled multigene cassettes for their use in transformation of the *Chlamydomonas* nuclear genome. Final vector design is detailed in Table 2 at the end of the document, and new level 1 cassettes were constructed accordingly. Due to the relatively high number of genes included in some of the level 2 vectors, these were designed to contain two antibiotic resistance genes (for bleomycin and paromomycin) flanking both sides of the constructs. Dual selection would therefore ensure introduction of both ends of the cassette in the genome and minimize the number of selected colonies with loss of cassette fragments. The use of riboswitch parts in some of the level 1 cassettes decreased the assembly efficiency possibly due to its three-dimensional structures. We therefore used commercial NEB5α stable competent cells. Level 2 assemblies also proved themselves challenging probably due to vector complexity and had to be attempted through continuous repetitions and the optimization of part concentrations in some cases. All level 1 plasmids were checked by sequencing, and level 2 plasmids were checked by digestion with restriction enzymes (Figure 2) and sequencing of the cassette flanking regions.

See Table 1 for complete list of generated vectors.

(4) Transformation of *Chlamydomonas reinhardtii*

C. reinhardtii strain uvm4 [2] has already been transformed by electroporation with the developed vectors. Transformants will undergo selection by growth in TAP media containing zeocin and paromomycin.

(5) Strain characterization and riboswitch activation and tuning

After transformation of *C. reinhardtii* the following scenarios can be encountered:

(5.1) Pigmented colonies appear.
Gene presence and expression levels will be assessed by PCR and qPCR. Betalain production and composition in transgenic *C. reinhardtii* lines will be analysed using a spectrophotometer by measuring absorbance of betacyanins and betaxanthins at 540nm and 475nm respectively. Experiments to assess riboswitch ability to direct metabolic flux to either branch of the betalain pathway will be performed once the transgenic lines have been identified.

(5.2) Transformed C. reinhardtii strains don't produce any coloration.
Gene presence on resistant colonies can be assessed by PCR to confirm integration of all the parts of our multigene cassette. In the cases where all genes are present, expression levels can be quantified by qPCR to rule out silencing of one or several of the transgenes. If these are as expected, new constructs with tagged versions of our transgenes should be designed to confirm protein presence and localisation.

(5.3) No colonies survive the selection process.
Transformation of *C. reinhardtii* would be repeated to rule out possible experimental errors. It is possible that one or more intermediates of the betalain pathway, or even pigment concentration itself, could have toxic effects on microalgae. To account for this possibility, transformation of *C. reinhardtii* with our riboswitch containing vector could be repeated, but this time adding thiamine to the selection media. This would inactivate the pathway hence allowing for a normal development of resistant colonies. Later, the colonies would be subcultured into liquid media containing a reduced concentration of thiamine that allows basal level of gene expression and thereby allow the optimization of expression ratios that would overcome potential cytotoxicity.

Expenditures

Item	Supplier	Price (£)	Quantity	Catalog n	Total price (+VAT)	Date
Gene synthesis with GeneArt (OpenPlant)	ThermoFisher Scientific	1517,43	1	PD2627147	1820,92	12/10/2017
OpenPlant Oligos for new Promoters	IDT	94,98	1	PD2644503	113,98	06/11/2017
PCRBIO HiFi Polymerase, 200 Units	PCR Biosystems	69	1	PB10.41-02	82,8	04/12/2017
Macherey Nagel, NucleoSpin Plasmid, 250preps	Macherey Nagel	149,95	1	740588250	179,94	22/2/2018
OpenPlant Oligos for sequencing	IDT	23,94	1	PD2675116	28,73	20/12/2017
OpenPlant Oligos for sequencing 2	IDT	3,24	1	PD2690119	3,89	22/01/2018
NEB® Stable Competent E coli (High Efficiency)	NEB	164,8	1	C3040I	197,76	22/01/2018
Gene Pulser/MicroPulser Cuvette, 0.4 cm gap, sterile, bag/50	Bio-Rad	114,95	1	1652088	137,94	22/02/2018
Sequencing reactions	Source Biosciences	4,5	94		507,60	multiple
TOTAL					3073,55	
					SPENT (£)	**LEFT (£)**
					3073,55	926,45
Expected future expenses	Cost / element	**Price (£)**				
RNEasy Plant Mini Kit (2x)	£ 209,85 / 50 rxn	419,7				
SuperScript III Full Kit	£516.8 / 50 rxn	516,8				
TOTAL		936,5				

Follow on plans

Following the selection process, betalain producing *C. reinhardtii* strains will be characterized and riboswitch efficacy to control protein ratios and ultimately the direction of metabolic flux towards red or yellow coloration will be assessed.

In order to facilitate the downstream analysis of the betalain compounds produced, extra funds provided would contribute to the purchase of a ZORBAX Eclipse XDB-C18 (5μm, 4.6 x 50 mm) column for its use in liquid chromatography (LC-MS) quantification and analysis.

The isolated FDX1 terminator contained an undomesticated *BpiI* site which interfered with the correct assembly of Level 2 cassettes. Extra funding could also be used for the synthesis of this part and RPL35 promoter and terminator regions in the case further cloning attempts were unsuccessful.

ZORBEX Eclipse XDB-C18 (5μm , 4.6 x 50 mm) – currently £257.55 in the University MarketPlace.

References

[1] López-Paz C, Liu D, Geng S and Umen JG. 2017. Identification of Chlamydomonas reinhardtii endogenous genic flanking sequences for improved transgene expression. *Plant J*, 92: 1232–1244.

[2] Neupert J, Karcher D, Bock R. 2009. Generation of Chlamydomonas strains that efficiently express nuclear transgenes. *The Plant Journal,* 57: 1140–1150.

Table 1. Complete list of generated level 0, level 1 and level 2 vectors compatible with the MoClo Golden Gate cloning technology.

Level 0

Vector ID	Backbone		Resistence	Level 0 / Entry			
				5' overhang	Contains	Species	3' overhang
pL0-ChcoDODAa	GeneArt backbone	CDS	Kanamycin	AATG	DODAa1	*Chlamydomonas reinhardtii*	GCTT
pL0-ChcoCYP76AD1	GeneArt backbone	CDS	Kanamycin	AATG	CYP76AD1	*Chlamydomonas reinhardtii*	GCTT
pL0-ChcoCYP76AD6	GeneArt backbone	CDS	Kanamycin	AATG	CYP76AD6	*Chlamydomonas reinhardtii*	GCTT
pL0-ChcocDOPA5GT	GeneArt backbone	CDS	Kanamycin	AATG	cDOPA5GT	*Chlamydomonas reinhardtii*	GCTT
pL0ATOP1	pICH41295	Promoter	Spectinomycin	GGAG	RPL23 Promoter	*Chlamydomonas reinhardtii*	TACT
pL0ATOP2	pICH41233	Promoter-Ntag	Spectinomycin	GGAG	RPL23 Promoter	*Chlamydomonas reinhardtii*	AATG
pL0ATOP5	pICH41295	Promoter	Spectinomycin	GGAG	FDX1 Promoter	*Chlamydomonas reinhardtii*	TACT
pL0ATOP6	pICH41233	Promoter-Ntag	Spectinomycin	GGAG	FDX1 Promoter	*Chlamydomonas reinhardtii*	AATG
pL0ATOP7	pICH41276	Terminator	Spectinomycin	GCTT	RPL23 Terminator	*Chlamydomonas reinhardtii*	CGCT
pL0ATOP8	pICH41276	Terminator	Spectinomycin	GCTT	FDX1 Teminator	*Chlamydomonas reinhardtii*	CGCT

Level 1

Vector ID	Backbone		Resistence	Level 1 / Cassette (co = codon optimised)				
				Promoter	NT-tag	CDS	CT-tag	Terminator
pL1ATBleR	pICH47802	position 1 rev	Ampicillin	PSAD		Bleomycin R		PSAD
pL1ATOP1	pICH47742	position 2 fwd	Ampicillin	FDX1		ChcoDODAa1		FDX1
pL1ATOP2	pICH47751	position 3 fwd	Ampicillin	PSAD	Riboswitch1	ChcoCYP76AD6		PSAD
pL1ATOP3	pICH47761	position 4 fwd	Ampicillin	HSP70::RBCS2	Riboswitch2	ChcoCYP76AD1		CA1
pL1ATOP4	pICH47751	position 3 fwd	Ampicillin	HSP70::RBCS2	RBCS2(i)	ChcoCYP76AD6		CA1
pL1ATOP5	pICH47761	position 4 fwd	Ampicillin	HSP70::RBCS2	RBCS2(i)	ChcoCYP76AD1		CA1
pL1ATOP6	pICH47772	position 5 fwd	Ampicillin	RPL23		ChcocDOPA5GT		RPL23
pL1ATOP9	pICH47742	position 2 fwd	Ampicillin	FDX1		ChcoDODAa1		RPL23

Level 2

Vector ID	Backbone	Resistence	Level 2					
			position 1	position 2	position 3	position 4	position 5	position 6
pL2ATOP1 (pBET)	pICSL4723	Kanamycin	PSAD/BleR/PSAD (rev)	FDX1/ChcoDODA/RPL23	PSAD/Riboswitch1CYP76AD6/PSAD	HSP70::RBCS2/Riboswitch2/CYP76AD1/CA1	RPL23/ChcocDOPA5GT/RPL23	HSP70::RBCS2/ParR/RBCS2
pL2ATOP3 (pBCY+)	pICSL4723	Kanamycin	PSAD/BleR/PSAD (rev)	FDX1/ChcoDODA/RPL25	PSAD/CTP/ChcoADHa/PSAD	HSP70::RBCS2/RBCS2(i)/CYP76AD1/CA1	RPL23/ChcocDOPA5GT/RPL23	HSP70::RBCS2/ParR/RBCS2
pL2ATOP5 (pBTX+)	pICSL4723	Kanamycin	PSAD/BleR/PSAD (rev)	FDX1/ChcoDODA/RPL27	HSP70::RBCS2/RBCS2(i)/CYP76AD6/CA1	PSAD/CTP/ChcoADHa/PSAD	Dummy	HSP70::RBCS2/ParR/RBCS2

Table 2. Detailed level 2 design for each of the constructed vectors. pBET is the vector containing riboswitches for CYP76AD1 and CYP76AD6. pBCY and pBTX are vectors designed to produce betacyanins (red) and betaxanthins (yellow) respectively.

Vector ID		Position 1			Position 2			Position 3				Position 4				Position 5			Position 6		
		Ter	CDS	Pro	Pro	CDS	Ter	Pro	RS	CDS	Ter	Pro	RS	CDS	Ter	Pro	CDS	Ter	Pro	CDS	Ter
pBET	pL2ATOP1	PSAD	BleR	PSAD	FDX1	DODA	RPL23	PSAD	Riboswitch1	CYP76AD6	PSAD	HSP70:RBCS2	Riboswitch2	CYP76AD1	CA1	RPL23	cDOPA5GT	RPL23	HSP70:RBCS2	PaR	RBCS2
pBCY	pL2ATOP2	PSAD	BleR	PSAD	FDX1	DODA	RPL23	Dummy				HSP70:RBCS2	intron	CYP76AD1	CA1	RPL23	cDOPA5GT	RPL23	HSP70:RBCS2	PaR	RBCS2
pBTX	pL2ATOP4	PSAD	BleR	PSAD	FDX1	DODA	RPL23	HSP70:RBCS2	intron	CYP76AD6	CA1	Dummy				Dummy			HSP70:RBCS2	PaR	RBCS2

Figure 1. PCR performed to isolate 5’ and 3’ flanking regions for RPL23, RPL35 and FDX1 from *C. reinhardtii* genomic DNA. A, promoter region using primers with 3’ overhang TACT; B, promoter region using primers with 3’ overhang AATG; Ter, terminator region. White squares indicate right sized bands.

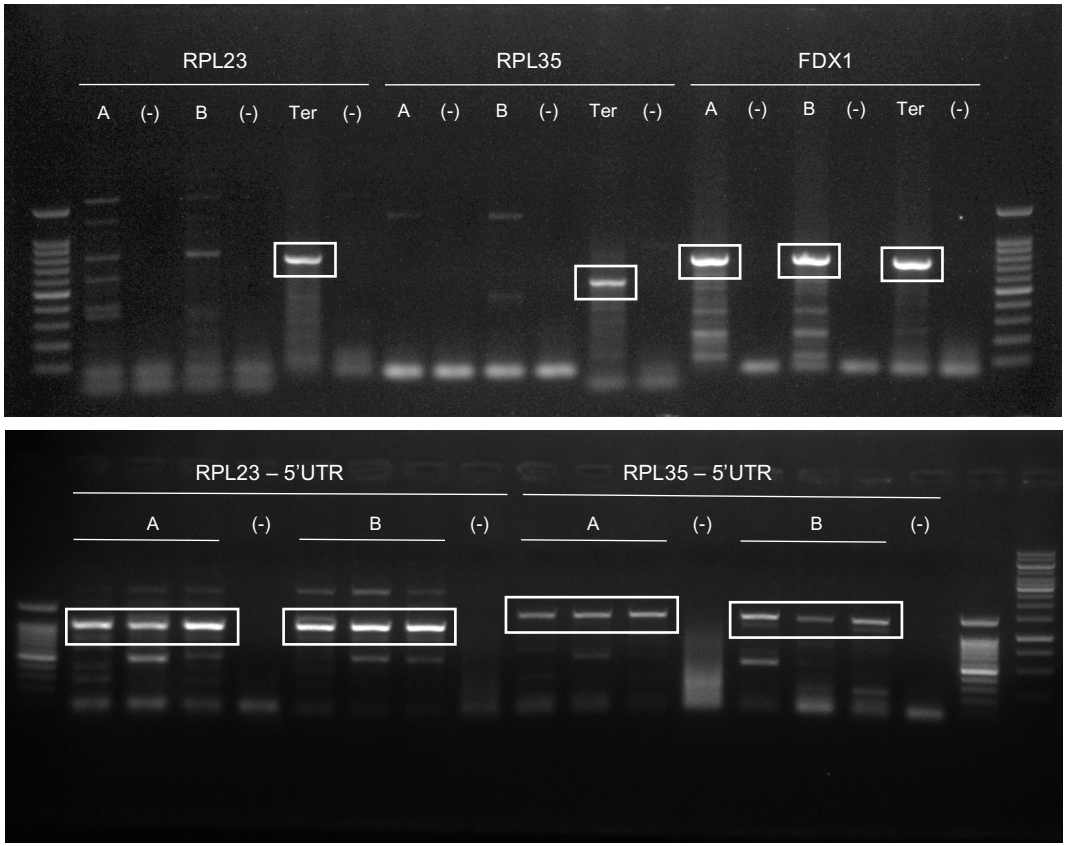

Figure 2. Diagnostic digestions for level 2 vectors. Numbers correspond to different plasmid extracted from independent colonies. pBET and pBCY were digested with *NheI*. pBTX was digested with *EcoRI*. Asterisks correspond to correct expected digestion patterns.