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Proposal Title

Implementation of a synthetic transcriptional AND gate in the chloroplast of *Chlamydomonas reinhardtii*

The Idea

Chloroplasts are among the most attractive substrates for biological engineering. Compared to nuclear transformation, integration of a transgene into the chloroplast genome comes with a number of notable advantages: i) great precision of genetic engineering due to highly efficient homologous recombination, ii) the opportunity to stack multiple transgenes within synthetic operons, iii) the absence of mechanisms for epigenetic transgene silencing, iv) immense levels of overexpression attainable (Oey et al. 2009), and v) increased biosafety due to the effective absence of chloroplasts from pollen (Bock 2015). Despite these features, the chloroplast has to date not been widely utilized as a platform for metabolic engineering. One of the major limitations to realization of its potential has been a lack of suitable systems for controlling the expression of transgenes from the chloroplast genome. Over the past decade, several conditional expression systems inducible by IPTG (Mühlbauer and Koop 2005), ethanol (Lossl 2005), and theophylline (Verhounig et al. 2010) have been developed. However, all of these systems respond to a single input only. In order to enable more sophisticated control over chloroplast gene expression based on multiple conditions, we propose to develop a synthetic transcriptional AND gate in the chloroplast of *Chlamydomonas reinhardtii*. *C. reinhardtii* was chosen as model organism to host the proof-of-concept circuit due to its relative ease of chloroplast-nuclear co-transformation (Ramundo et al. 2013).

The nuclear component of the proposed circuit is composed of two chloroplast-targeted halves of split T7 RNA polymerase (T7RNAP; Shis and Bennett 2013) which are conditionally expressed under control of two different input promoters. Co-induction of the two polymerase halves targeted to the chloroplast will lead to their spontaneous

association to form a functional enzyme which will drive expression of a chloroplast-encoded transgene from a T7 promoter. In scope of the proof of concept proposed here, this transgene will take the shape of a fluorescent reporter quantified in a ratiometric manner (Rudge et al. 2015) to assess functionality of the genetic circuit. In the future, this fluorescent output will be replaced by a transgene modifying secondary metabolism in response to two different input signals.

Briefly summarise your proposal

Who We Are

1. Christian R. Boehm

Doctoral Researcher, Department of Plant Sciences, University of Cambridge

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I am a synthetic biologist working on the development of genetic circuits controlling transgene expression in chloroplasts. My expertise relevant to this proposal embraces the design and ratiometric characterization of a synthetic transcriptional AND gate in *E.* coli, the development of T7RNAP-driven transgene expression in *M. polymorpha*, and transformation of chloroplasts in *Marchantia polymorpha* by fluorescent reporters.

2. Payam Mehrshahi

Postdoctoral Scientist, Department of Plant Sciences, University of Cambridge

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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.

3. Hannah Laeverenz-Schlogelhofer

Doctoral Researcher, Department of Physics, University of Cambridge
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I am interested in microbial biophysics, cellular interactions and metabolism. Particularly relevant to this proposal is the work that I have done in developing image analysis tools for quantifying fluorescent reporters in algae, which I have so far used to study the vitamin B_{12} dependent METE promoter in *Chlamydomonas reinhardtii.* The use of my image analysis script will benefit the proposed work by offering quantitative analysis of fluorescent reporters from confocal micrographs.

Names and background of interdisciplinary team (include email addresses)

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Implementation

This proposal embraces the following milestones:

I. Synthesis of codon-optimized T7RNAP and fluorescent reporter genes

Transcriptional activity of a T7RNAP split between amino acid residues 179 and 180 has been verified in *E. coli* by means of a ratiometric assay prior to submission of this proposal (C.R. Boehm, unpublished results). It was found that splitting the enzyme led to an increase in relative activity from the T7 promoter by four-fold compared to the intact enzyme due to reduction of background transcription. We will design and order a *t7rnap* gene whose coding sequence has been optimized to reflect codon usage in the nuclear genome of *C. reinhardtii* (Merchant et al. 2007). We will also design and order spectrally separated fluorescent reporter genes *mvenus* (Nagai et al. 2002) and *mturquoise2* (Goedhart et al. 2012; Boehm et al. 2015) whose coding sequences have been optimized to reflect codon usage in the chloroplast genome of *C. reinhardtii* (Maul et al. 2002).

II. Introduction of a ratiometric reporter into the chloroplast genome of *C. reinhardtii*

We will adapt the design of a ratiometric reporter cassette responsive to T7RNAP activity, which has previously been implemented into the chloroplast genome of *Marchantia polymorpha* (C.R. Boehm, unpublished results), to the chloroplast genome of *C. reinhardtii*. The ratiometric reporter cassette in question embraces a codon-optimized *mvenus* yellow fluorescent reporter gene under control of the T7 promoter, and a codon-optimized *mturquoise2* cyan fluorescent reporter gene under control of a constitutive promoter. We will modify the existing design by i) replacing the fluorescent reporter genes with variants optimized to reflect codon usage in the *C. reinhardtii* chloroplast genome (see I.), ii) replacing the constitutive promoter controlling *mturquoise2* in the original design by means of the psaA-exon1 leader proven in *C. reinhardtii* (Michelet et al. 2011), iii) addition of an erythromycin resistance gene as selectable marker (Kindle et al. 1991), and iv) flanking the ratiometric reporter cassette by sequences for homologous recombination into the the *C. reinhardtii* chloroplast genome via of the proven psbA site (Manuell et al. 2007). Transformation of the *C. reinhardtii* chloroplast genome will be performed as previously reported (Ramesh et al. 2011). Following antibiotic selection, successful transformants will be confirmed by confocal microscopy via screening for constitutively expressed cyan fluorescent protein. Fluorescence intensities will be automatically extrapolated from confocal micrographs by means of a code adapted from a MATLAB feature tracking script, which has been created by the Kilfoil lab (http://people.umass.edu/kilfoil/downloads.html). Features are identified by fitting Gaussian intensity profiles to the transmission bright field image, which are confirmed to be algal cells using their chlorophyll autofluorescence. The fluorescence image is background corrected and using the determined cell positions and sizes, the sum of the intensity within each cell area is obtained to give the fluorescence intensity per cell. Finally, homoplasmy and integrity of the transgene cassette introduced into the chloroplast genome of *C. reinhardtii* will be verified by analytical PCR.

III. Implementation of a synthetic transcriptional AND gate in the chloroplast of *C. reinhardtii*

Transplastomic *C. reinhardtii* encoding a ratiometric reporter cassette in their chloroplast genome (see II.) will be subjected to nuclear transformation (Shimogawara et al. 1998) for implementation of the synthetic transcriptional AND gate. Five different versions of the nuclear transformation vector will be employed, encoding codon-optimized (see I.) i) intact T7RNAP, ii) N-terminal half of split T7RNAP only, iii) C-terminal half of split T7RNAP only, iv) N- and C-terminal halves of split T7RNAP controlled by one constitutive and one inducible promoter, respectively, and v) a variant of the former construct with the constitutive and inducible promoters swapped. This set of vectors is sufficient for validation of functional Boolean AND logic. The proven PsaD (Fischer and Rochaix 2001) and METE (Helliwell et al. 2014) promoters will be used to drive expression of T7RNAP genes in a constitutive or inducible manner, respectively. T7RNAP or its fragments will be targeted to the chloroplast of *C. reinhardtii* by means of a transit peptide derived from the N-terminal region of PsaD (Fischer and Rochaix 2001).

Describe what you are planning to do with the funding, including aims, methods, outcomes and who will be involved.

Benefits and outcomes

The proposed interdisciplinary project brings together expertise in the development of genetic logic gates (C.R. Boehm, Haseloff group, Department of Plant Sciences), transformation of the nuclear and chloroplast genomes of *C. reinhardtii* (P. Mehrshahi, Smith group, Department of Plant Sciences), and the automated extrapolation of fluorescence intensity from confocal micrographs of algae (H. Laeverenz-Schlogelhofer, Croze group, Department of Physics), thereby promoting knowledge exchange between different Departments in Cambridge. In addition, we are actively looking for collaborators from both the Sainsbury Laboratory and the John Innes Centre Norwich who may be interested in extending the logic circuits developed herein for control of transgene expression in Nicotiana or other angiosperm models. Due to the high degree of

conservation between chloroplast genomes, we expect the input promoters driving expression of the nucleus-encoded, chloroplast-targeted polymerase fragments to be the only circuit components requiring replacement upon adoption of the system to a different model species. On basis of substantial preliminary work conducted over the past two years, the success of our project will generate high-profile publishable results within a relatively short amount of time, and will be seen as a pioneering effort in the field of chloroplast biotechnology.

Describe how your project fits the remit of OpenPlant and the judging criteria, including details of any new interdisciplinary interactions between Cambridge and Norwich.

Sponsor for the research and cost centre

Prof. Jim Haseloff

Director, OpenPlant Synthetic Biology Research Center

Department of Plant Sciences, University of Cambridge

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Name, institution, department and email address of the individual who will support the project and sponsor the cost-code to which you want funds to be allocated. This would generally be a research supervisor.

Sponsor support confirmed?

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).

Budget

Technique	Cost/sample	No. of samples	Total £
DNA synthesis for coding genes	£0.09/bp	12,276 bp	1,104.84
Sequencing	£4.95/reaction	500	2,475
Phire Plant Direct PCR Kit	£0.36/reaction	1000	360
Total			3939.84

Provide costings for your proposal (up to £4000) and indicate if you have access to any additional funding to meet your aims.

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