

# Six Months Report: Implementation of a synthetic transcriptional AND gate in the chloroplast of *Chlamydomonas reinhardtii*

## Summary

The chloroplast is among the most attractive substrates for biological 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 have been developed. However, all of these systems have been designed responsive to a single input only. In order to enable more sophisticated control over chloroplast gene expression based on multiple conditions, we proposed to develop a synthetic transcriptional AND gate implemented in the chloroplast of *Chlamydomonas reinhardtii*, based on a modified T7 bacteriophage RNA polymerase (T7RNAP). We designed optimized yellow and cyan fluorescent reporters reflecting codon usage in the *C. reinhardtii* chloroplast genome, and an optimized T7RNAP gene reflecting codon usage in the *C. reinhardtii* nuclear genome. We have domesticated the optimized genes to be compatible with Golden Gate assembly. We have designed nuclear transformation vectors encoding intact and split T7RNAP variants under control of constitutive expression signals or riboswitches. We have further attempted introduction of the optimized fluorescent reporters into the *C. reinhardtii* chloroplast genome, and are waiting to confirm establishment of homoplasmy prior to demonstration of AND gate functionality.

## Report and Outcomes:

We were awarded an OpenPlantFund grant to implement a synthetic transcriptional AND gate based on a modified split T7RNAP into the chloroplast of the green alga *C. reinhardtii*. This proposal embraced the following milestones: (i) the synthesis of codon-optimized T7RNAP and fluorescent reporter genes, (ii) the introduction of a ratiometric reporter into the chloroplast genome of *C. reinhardtii*, and (iii) the implementation of a synthetic transcriptional AND gate in the chloroplast of *C. reinhardtii*. We have since been joined by Aleix Gorchs-Rovira from the Alison Smith group, who contributes valuable experience in *C. reinhardtii* chloroplast transformation to our team.

We completed milestone (i) by design and synthesis of optimized fluorescent reporter genes *cpmVenus* (YFP) and *cpmCerulean3* (CFP) adapted to codon usage of the chloroplast genome of *C. reinhardtii*. We also domesticated these genes for Golden Gate assembly by removing internal *BsaI*, *BsmBI*, and *BpiI* restriction sites. As part of milestone (ii), we proposed to use both fluorescent reporter genes to design a transformation vector enabling ratiometric characterization of gene expression from the *C. reinhardtii* chloroplast genome. To this end, we first sought to validate expression of both *cpmVenus* and *cpmCerulean3* from the chloroplast genome of *C. reinhardtii*. We designed ten different variants of a chloroplast transformation vector where the fluorescent proteins were controlled by different expression signals:

(promoter:5'UTR:CDS:3'UTR/Terminator)

- *psaA:psaA:cpmVenus/cpmCerulean3:psbA* (for constitutive expression)
- *psbD:psbD:cpmVenus/cpmCerulean3:psbA* (for high, light-dependent expression)
- *16S:atpA:cpmVenus/cpmCerulean3:psbA* (for very high expression)
- *T7:T7:cpmVenus/cpmCerulean3:T7* (for T7RNAP-dependent expression)
- *T7:atpA:cpmVenus/cpmCerulean3:T7* (for T7RNAP-dependent expression using native translational enhancers)

We constructed the transformation vectors using Golden Gate assembly at Level 2. In addition to respective reporter expression cassettes outlined below, the transformation vectors each contained a kanamycin resistance cassette and flanking regions for homologous recombination into the *psbA* intergenic region of the *C. reinhardtii* chloroplast genome. We introduced all ten transformation vectors into *C. reinhardtii* using particle bombardment, and recovered kanamycin-resistant colonies. Unfortunately, putative transformants died during iterative propagation for establishment of homoplasmy due to a failure in nutrient supply. We aim to complete repeat biolistic transformations before end of this month.

In parallel, we prepared the nuclear component of the synthetic transcriptional AND gate to be introduced as part of milestone (iii) into transplastomic *T7:cpmVenus/cpmCerulean3 C. reinhardtii* following confirmation of their homoplasmy and detectable baseline expression *cpmCerulean3*. We designed and synthesized an optimized *T7RNAP* gene adapted to codon usage of the nuclear genome of *C. reinhardtii*. Based thereupon, we designed five different variants of a nuclear transformation vector encoding externally inducible T7RNAP targeted to the *C. reinhardtii* chloroplast:

- *pPSAD:RBCS2 Intron:cTP-PSAD:CrT7RNAP:CA1 3'UTR* (for constitutive expression of chloroplast-targeted T7RNAP)
- *pPSAD:RBCS2 Intron:cTP-PSAD:N-Term\_CrT7RNAP: CA1 3'UTR* (for constitutive expression of chloroplast-targeted N-terminal half of split T7RNAP)

- *pPSAD:RBCS2 Intron:cTP-PSAD:C-Term\_CrT7RNAP: CA1 3'UTR* (for constitutive expression of chloroplast-targeted C-terminal half of split T7RNAP)
- *pPSAD:RBCS2 Intron:CrTHIC\_RS:cTP-PSAD:N-Term\_CrT7RNAP: CA1 3'UTR* (for riboswitch-regulated expression of chloroplast-targeted N-terminal half of split T7RNAP)
- *pPSAD:RBCS2 Intron:CrTHI4\_RS:cTP-PSAD:C-Term\_CrT7RNAP: CA1 3'UTR* (for riboswitch-regulated expression of chloroplast-targeted C-terminal half of split T7RNAP)
- *pPSAD:RBCS2 Intron:CrTHIC\_RS:cTP-PSAD:N-Term\_CrT7RNAP: CA1 3'UTR*
- *pPSAD:RBCS2 Intron:CrTHI4\_RS:cTP-PSAD:C-Term\_CrT7RNAP: CA1 3'UTR* (for riboswitch-regulated expression of chloroplast-targeted N- & C-terminal halves of split T7RNAP)

We synthesized or PCR-amplified all parts for the above constructs to carry the MoClo syntax and be compatible with Golden Gate assembly. We expect introduction of these vectors into transplastomic *T7:mVenus/mCerulean3* to produce expression of the fluorescent reporter from the chloroplast genome only under presence of chloroplast-targeted intact CrT7RNAP, or under coincidence of the chloroplast targeted N-terminal and C-terminal CrT7RNAP fragments. The latter experiment will serve as demonstration of chloroplast-encoded Boolean AND logic in *C. reinhardtii*.

All vectors designed and constructed as part of this project will be made openly available via Genbank and Addgene within a few months' time as the vector backbones used in this project have been published as part of separate work conducted under supervision of Alison Smith. Meanwhile, we have made the optimized genes *cpmVenuscp*, *cpmCerulean3cp*, and *CrT7RNAP* available via Genbank accession numbers KY484011, KY484012, and KY484013, respectively

## Expenditure:

Item	Cost (£)	Balance
Custom gene synthesis	1,231.26	+ 2768.74
DNA oligonucleotides	158.42	+2610.32
Sequencing	495.00	+2115.32

# Follow On Plans

We would like to request £1,000 of follow-on funding for synthesis of three additional promoters (1,300bp each) and of four additional terminators (1,000bp each) to increase our capacity for controlling expression of split T7RNAP from the nuclear genome of *C. reinhardtii*. According to IDT rates, the cost for this undertaking is projected at £980.