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Letter

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Birth of a photosynthetic chassis: a MoClo toolkit enabling synthetic biology in the

microalga *Chlamydomonas reinhardtii*

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27   **Abstract**

28   Microalgae are regarded as promising organisms to develop innovative concepts based on

29   their photosynthetic capacity that offers more sustainable production than heterotrophic

30   hosts. However, to realize their potential as green cell factories, a major challenge is to make

31   microalgae easier to engineer. A promising approach for rapid and predictable genetic

32   manipulation is to use standardized synthetic biology tools and workflows. To this end we

33   have developed a Modular Cloning toolkit for the green microalga *Chlamydomonas*

34   *reinhardtii*. It is based on Golden Gate cloning with standard syntax, and comprises 119

35   openly distributed genetic parts, most of which have been functionally validated in several

36   strains. It contains promoters, UTRs, terminators, tags, reporters, antibiotic resistance genes,

37   and introns cloned in various positions to allow maximum modularity. The toolkit enables

38   rapid building of engineered cells for both fundamental research and algal biotechnology.

39   This work will make *Chlamydomonas* the next chassis for sustainable synthetic biology.

40

41   **Keywords**: Algal biotechnology, *Chlamydomonas reinhardtii*, modular cloning, synthetic

42   biology.

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45 There is an urgent need to decarbonize the world economy due to depletion of fossil fuel

46 reserves coupled with accumulation of greenhouse gases produced by their combustion.

47 One alternative to the use of fossil fuels is to use photosynthetic microorganisms, such as

48 microalgae, as green cell factories to produce fuels and chemicals from atmospheric CO_2 in a

49 sustainable process driven by sunlight[1, 2]. The fixed carbon can be redirected towards

50 compounds that can be used in the fuel, food, cosmetic and pharmaceutical industries, such

51 as proteins, alcohols, alkanes, lipids, sugars, pigments or terpenes[3-5]. By contrast with land

52 plant-based photoproduction, microalgae do not compete with agriculture and can be grown

53 at high yields even at large scale[4, 6], including on waste streams, thus minimizing inputs[3]. The

54 green microalga *Chlamydomonas reinhardtii* (referred to hereafter as "Chlamydomonas")

55 has been extensively engineered for basic research and industrial biotechnology[4, 6-8]. Its

56 nuclear and organellar genomes are sequenced and annotated, molecular biology

57 techniques and culture conditions are highly developed, and its physiology and metabolism

58 are well understood[9-13]. Moreover, the metabolic plasticity and cellular compartments of

59 Chlamydomonas offer great potential for advanced metabolic engineering strategies[14, 15].

60 Chlamydomonas has already been engineered for production of the biodiesel precursor

61 bisabolene[8], the terpene patchoulol[7], and recombinant proteins as well as enzymes such as

62 an HIV antigen[16] and xylanase[17]. Despite these proofs of concept however, engineering of

63 Chlamydomonas is still slow due to a lack of standardized resources and tools[11].

64 Development of the field of algal synthetic biology offers the means to enable design and

65 construction of microalgal cells with defined and predictable properties[18]. Besides

66 biotechnological applications, the transition from empirical to synthetic approaches also

67 provides the opportunity to answer fundamental biological questions using new concepts

68 and approaches based on understanding by construction rather than deconstruction.

3

69   Synthetic biology approaches, predicated on the Design-Build-Test-Learn cycle[19], make

70   organisms easier to engineer through the use of standardized parts and their assembly to

71   simplify the building of designed DNA molecules[19]. Among available standards[20], the Golden

72   Gate Modular Cloning (MoClo) technology, based on Type IIS restriction enzymes, offers

73   extensive standardization and allows the assembly of complex multigenic DNA from basic

74   gene parts (*e.g.* promoters, CDS, terminators) in just two steps[21, 22]. The method accelerates

75   and multiplies the possibilities to permute multiple genetic elements, and makes facile the

76   building of multigene constructs for full metabolic pathways[23]. MoClo is efficient and

77   versatile, but relies on intensive upfront generation of a standardized library of basic

78   building blocks, the gene parts, that have been domesticated to remove Type IIS sites, and

79   codon optimized for the host as appropriate. MoClo toolkits have already been developed

80   for a few model organisms[24-29] although not yet for microalgae.

81   Here, we report the generation of a MoClo toolkit composed of more than 100 gene parts

82   codon-optimized for the Chlamydomonas nuclear genome. These genetic parts were

83   designed to provide maximum modularity to end-users, and to facilitate the development of

84   engineered strains for fundamental and green biotechnological applications, through

85   iterative design and testing. We provide functional validation and characterization of many

86   gene parts in several Chlamydomonas strains. This kit is available to the community, to allow

87   Chlamydomonas to become the next chassis for sustainable synthetic biology approaches.

88   **RESULTS**

89   **Standard and content of the Chlamydomonas MoClo kit**

90   Standardization is the key to efficient building. The Chlamydomonas MoClo kit adopts the

91   syntax proposed by the plant synthetic biology community including the OpenPlant

4

92   Consortium[30] (Fig. 1). This syntax is defined for level 0 plasmids containing standard gene

93   parts (promoters, coding sequences, untranslated regions, etc.) and assigns strict fusion sites

94   for 10 cloning positions. In a single step, standardized parts can be assembled into modules

95   (Transcriptional Unit, TU, level 1) and modules into devices (multigenic construct, level M or

96   2) according to the original MoClo syntax[22] (Supplementary Fig. 1). Our Chlamydomonas

97   MoClo toolkit is composed of a set of 119 parts representing 67 unique genetic elements

98   available at different positions within the standard, thereby providing maximum modularity

99   to designers (Fig. 1, Fig. 2). The kit recapitulates most of the standard genetic elements

100   previously developed for Chlamydomonas which we "domesticated" by removing *Bpi*I and

101   *Bsa*I restriction sites (the two enzymes used by the MoClo strategy[22], Supplemental Figure 1)

102   from their sequences by DNA synthesis or PCR-based mutagenesis. The available gene parts

103   encompass 7 promoters coupled or not to their original 5'UTR, the corresponding 5'UTR and

104   the *CrTHI4* riboswitch, 8 immunological or purification tags in positions leading to N- or C-

105   terminal translational fusions, 9 signal and targeting peptides, 12 reporters, 5 antibiotic

106   resistance genes, the foot and mouth virus (FMDV) 2A peptide which allows expression of

107   two or more proteins from a single transcriptional unit[17, 31, 32], 2 micro RNA (miRNA)

108   backbones and associated controls, and six 3'UTR-terminators (Fig. 1b, Fig. 2 and

109   Supplementary Table 1). All sequences and plasmids are available through the public

110   Addgene repository (http://www.addgene.org/).

111   **Constitutive promoters and reporter genes**

112   Five antibiotic resistance genes are used as selectable markers for Chlamydomonas but also

113   can function as reporter genes[33, 34]. We assembled three modules that allow control of the

114   expression of the *aadA* gene, conferring spectinomycin resistance, by three constitutive

5

115   promoters: P_{PSAD} and $P_{\beta TUB2}$ with or without the first intron of β*TUB2* (pCM1-1 to 3,

116   Supplementary Table 3). The transformation efficiency of the three modules in UVM4[35] cells

117   was estimated by counting spectinomycin resistant colonies and showed resistance

118   frequencies within the same range (Fig. 3a). The presence of the first β*TUB2* intron

119   significantly increased the transformation efficiency as previously observed with the

120   presence of *RBCS2* introns in the *ble* marker[33, 36, 37]. Alternative reporters are bioluminescent

121   proteins, which allow more sensitive and quantitative analysis of gene expression. The kit

122   contains *Gaussia princeps* luciferase, the brightest luciferase established in

123   Chlamydomonas[38], as well as the redesigned Nanoluciferase (NanoLuc) which provides a

124   stable and strong luminescence signal[39]. Chlamydomonas NanoLuc was specifically

125   developed for our MoClo kit through recoding to match the codon bias of Chlamydomonas,

126   and cloned at 6 different positions within the standard. This new part was first tested with

127   the most widely used promoter/terminator combination (P_{AR} promoter / T_{RBCS2} terminator)

128   for strong constitutive expression in Chlamydomonas. The corresponding module (pCM1-04)

129   was assembled with another module conferring paromomycin resistance (Supplementary

130   Fig. 2) into a device (pCMM-1) that was introduced into the genome of the D66 strain (CC-

131   4425, Fig. 3b). Among paromomycin resistant colonies, 34.8% ± 8.3 (N=48, mean ± SEM)

132   were luminescent. The signal was variable between clones due to genomic position effects[40,

133   [41] but was linear from 50 to 5×10^5 cells (Fig. 3b and Supplementary Fig. 2). By contrast, non-

134   expressing transformants (resistant to paromomycin only) or the D66 recipient strain

135   displayed only a faint signal, 3 orders of magnitude lower, and saturating swiftly (Fig. 3b,

136   inset). The modularity of the MoClo strategy allows rapid assessment of combinations of

137   multiple parts. For example, we assembled 4 modules where NanoLuc expression is

138   controlled by all possible combinations of the two most common constitutive promoters (P_{AR}

6

139   and P_{PSAD}) and terminators (T_{RBCS2} and T_{PSAD}) (Fig. 3b, pCM1-4 to 7, Supplementary Table 3).

140   Each module was assembled with the paromomycin resistance module (pCMM-1 to 4,

141   Supplementary Table 4) and introduced into the Chlamydomonas genome. Bioluminescence

142   levels were averaged over hundreds of transformants to account for the genome position

143   effect[40, 41]. The strengths of the two promoters were found to be comparable, whilst T_{PSAD}

144   appeared to confer robust expression from both promoters, 10-fold higher than T_{RBCS2} (Fig.

145   3c). In a distinct context (strain, reporter sequence, culture conditions, etc.), the same

146   genetic element may perform differently[31, 35]. Such context sensitivity can be overcome by

147   taking advantage of the modularity of the Chlamydomonas MoClo kit, which allows for the

148   rapid characterization of all possible parts combinations. These results also confirmed the

149   performance of the Chlamydomonas NanoLuc reporter and its employability for detailed

150   understanding and characterization of genetic circuits especially if coupled with automated

151   cell-sorting microfluidic devices[42].

152   **Control of gene expression**

153   To build genetic circuits, the fine-tuning of gene expression is a prerequisite. Multiple parts

154   enabling controlled gene expression have therefore been implemented. The activity of the

155   P_{NIT1} promoter can be controlled by switching the nitrogen source since it is strongly

156   repressed by ammonium and highly induced on nitrate[34, 43, 44]. A module where P_{NIT1} controls

157   expression of the *ble-GFP* gene (pCM1-8) conferred strong zeocin resistance in the CC-1690

158   strain but only when ammonium was replaced by nitrate as nitrogen source. By contrast, the

159   P_{PSAD} promoter (pCM1-9) conferred strong antibiotic resistance on both nitrogen sources

160   (Fig. 4a and Supplementary Fig. 3a-c). The vitamin B_{12}-repressible promoter P_{METE}[45] allowed

161   conditional functional complementation of the photosynthetic mutant *nac2-26* (CC-4421),

162 which lacks photosystem II due to the absence of the TPR-like protein NAC2 required for

163 stability of the *psbD* mRNA encoding the D2 reaction center protein[46]. *nac2-26* mutant cells

164 engineered with a module harboring the *NAC2* coding sequence under the control of the

165 P_{METE} promoter (pCM1-10) could grow photoautotrophically in the absence of vitamin B_{12},

166 but growth was compromised by increasing its concentration by amounts as low as 5 ng/L

167 (Fig. 4b).

168 Regulation of gene expression can also be controlled by vitamin B_1 (thiamine) at the level of

169 the transcript through riboswitches[47, 48]. Binding of thiamine pyrophosphate to the *THI4*

170 riboswitch (RS) results in alternative splicing and retention of an 81 bp upstream open

171 reading frame, ultimately interfering with translation[47, 48]. The RS also responds when cells

172 are grown in the presence of the thiamine biosynthetic intermediate 4-methyl-5-(2-

173 hydroxyethyl) thiazole (HET), but not with 4-amino-5-hydroxymethyl-2-methylpyrimidine

174 (HMP)[47]. A module combining P_{AR} and *THI4 (RS)* to drive expression of the *ble-GFP* gene

175 (pCM1-11) conferred conditional zeocin sensitivity in the UVM4 strain[35]. Resistance was

176 compromised by thiamine or HET but not HMP (Fig. 4c and Supplementary Fig. 3d), thereby

177 demonstrating the efficient repression of the transgene through the *THI4* riboswitch.

178 Finally, to allow targeted repression of gene expression, a microRNA precursor sequence

179 derived from the pre-miR1157 and used for the generation of artificial miRNAs (amiRNA)[49]

180 was re-designed for compatibility with the Golden Gate cloning method. To demonstrate its

181 effectiveness in driving gene repression, a specific amiRNA sequence directed against the

182 *MAA7* gene, whose repression provides resistance to 5'-fluoroindole (5'-FI)[50], was inserted

183 into the microRNA precursor. A control random sequence ("scrambled") amiRNA was

184 inserted into the same backbone. These parts were placed under the control of P_{PSAD} and

185 T_{PSAD} (pCM1-12 and 13) and assembled with a paromomycin resistance module (pCM1-27).

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186   The same amiRNA sequences were introduced into the previously established pChlamiRNA3

187   vector[49] as controls. After transformation of the CC-1690 strain, 36% of paromomycin-

188   resistant cells displayed resistance to 5'-Fl with the device targeting *MAA7* (pCMM-5) but

189   not with the scrambled amiRNA (pCMM-6) (Fig. 4d and Supplementary Fig. 3f). A modified 5'

190   rapid amplification of cDNA ends (5'-RACE) assay revealed that the *MAA7* transcript was

191   most frequently cleaved at a site corresponding to positions 10 and 11 of the amiRNA, as

192   expected for a specific action of the miRNA (Fig. 4d). The properties of controllable parts can

193   also be combined as shown for P_{NIT1} control of amiRNA-dependent gene repression[34]. An

194   amiRNA strategy recently proved useful for concerted metabolic engineering of a biodiesel

195   precursor in Chlamydomonas[8]. The versatility of the MoClo kit opens new possibilities for

196   sophisticated metabolic engineering strategies, *e.g.* the specific downregulation of up to six

197   target genes with one level M assembly.

198   **Multiple fusion tags for detection and purification of gene products.**

199   Protein fusion tags are indispensable tools used to improve protein expression yields, enable

200   protein purification, and accelerate the characterization of protein structure and function[51].

201   Our MoClo kit includes multiple epitope and affinity tags known to be functional in

202   Chlamydomonas. The modularity of the MoClo assembly allows rapid assessment of the best

203   tagging strategy through a rapid design/build/test/learn cycle. We took advantage of the

204   well characterized *rap2* mutant (*ΔFKBP12*), which is insensitive to rapamycin[52], to test the

205   functionality of five tags (Fig. 5a,b). We designed and built 5 devices allowing strong

206   constitutive expression of N- or C-terminal tagged FKBP12 coupled to a paromomycin

207   module (pCMM-7 to 11, Fig. 5c-h and Supplementary Table 4). The engineered strains were

208   selected on paromomycin and the functionality of the fusion protein was tested by assessing

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209   sensitivity to rapamycin. Protein extracts were probed by immunoblotting using FKBP12-

210   specific and tag-specific antibodies (Fig. 5d-h). All tags allowed detection (Fig. 5d-h) or

211   purification (Fig. 5i) of FKBP12 even though some were not functional for restoring

212   rapamycin sensitivity. The test revealed that pCMM-9 outperforms other devices since it

213   provides a WT-like phenotype and expression level coupled to a strong and specific Myc

214   signal with no significant processing of the protein. These results demonstrate the

215   importance of the modularity provided by the Chlamydomonas MoClo toolkit for designing

216   optimal fusion proteins.

217   **Visualization and targeting of proteins in living cells**

218   Fluorescent protein tags allow the temporal and spatial monitoring of dynamic expression

219   patterns at cellular and subcellular scales[53]. Natural and synthetic metabolic pathways can

220   be optimized through spatial organization since cell compartments offer many advantages,

221   such as isolation of metabolic reactions and generation of concentration gradients[14]. In a

222   eukaryotic chassis like Chlamydomonas, organelles such as microbodies, mitochondria and

223   chloroplasts can be engineered to implement or improve metabolic pathways[15]. The

224   Chlamydomonas MoClo kit includes 11 targeting and signal peptides that allow the targeting

225   of fusion proteins to mitochondria, chloroplast, nucleus, secretory pathway, ER and

226   peroxisome-like microbodies. The functionality of the targeting and signal peptides and of

227   the five fluorescent proteins (mVenus - yellow, mCherry - red, mRuby2 - red, Clover - green,

228   mCerrulean3 - cyan) included in the toolkit was tested. Eight modules (pCM1-19 to 26,

229   Supplementary Table 3) combining diverse fluorescent proteins and targeting sequences

230   were assembled into devices with an antibiotic resistance module (pCMM-12 to 19). All

231   devices were found to behave as expected and provided the expected fluorescent signal in

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232   the targeted compartment (Fig. 6). The fluorescent and targeting parts of the

233   Chlamydomonas MoClo toolkit, most of which have been validated here, enable engineering

234   in the third dimension[14] *i.e.* isolation and organization in multiple cellular compartments,

235   and offer new tools for biological design/build/test cycles.

Discussion

237   The Chlamydomonas MoClo toolkit presented here provides more than 100 domesticated

238   gene parts to allow advanced synthetic biology in microalgae. Numerous parts of multiple

239   types have been characterized and validated in different genetic backgrounds[10] and culture

240   conditions, and can be readily used for biological design without further development. With

241   the efficiency and modularity of the MoClo strategy, molecular cloning is no longer a limiting

242   step for engineering Chlamydomonas cells. Indeed, from design to building, a complex

243   device of up to six different genes/modules can be obtained within a week using the

244   standardized parts provided in our kit. The modularity will also enable combinatorial

245   assembly by shuffling part libraries[54] and determine *a posteriori* which combination is the

246   most relevant. The development of gene-editing technologies in Chlamydomonas, including

247   Zinc-finger nucleases[55, 56] and several CRISPR-Cas9 approaches[55, 57-59], together with the

248   development of high-throughput microfluidics[42] are beginning to gather pace. Coupling

249   these resources to our standardized MoClo toolkit will facilitate the use of Chlamydomonas

250   as the photosynthetic chassis for innovative synthetic biology approaches aimed at

251   fundamental and biotechnological applications. We expect that the creativity of designers,

252   released from the time constraints associated with classical cloning strategies, will allow

253   rapid expansion of the standard gene parts, modules and devices through open distribution,

254   notably using the Addgene repository. We invite the community to openly share their parts

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255   through Addgene and/or our consortium (contact M. Schroda). The development of the

256   Chlamydomonas MoClo toolkit constitutes a complete step-change in the fields of microalgal

257   biology and biotechnology. The parts developed for the MoClo toolkit may also be employed

258   in other microalgal species since the orthogonality of several Chlamydomonas

259   transcriptional units has been demonstrated in multiple hosts, including the industrially

260   relevant species *Chlorella ellipsoidea*, *Nannochloropsis sp.* and *Dunaliella salina*[60]. Synthetic

261   approaches will allow engineering of microalgae in a predictable and efficient manner and

262   thereby offer great potential to couple environmental protection, energy transition and

263   bioeconomic growth[4].

264

265   **Methods**

266   All chemicals were obtained from Sigma-Aldrich, unless otherwise specified.

267

268   ***Escherichia coli* and *Chlamydomonas reinhardtii* strains, transformation and growth**

269   **conditions.**

270   Bacterial growth was performed at 37°C in LB broth supplemented with agar (20% m/V),

271   spectinomycin (50 µg/mL), ampicillin or carbenicillin (50 or 100 µg/mL, respectively) and X-

272   gal (40 µg/mL) when required. Chemically competent *E. coli* DH10β (New England Biolabs)

273   were used for transformation (by heat shock following the manufacturer's instructions) and

274   maintenance of plasmids. All plasmids of the kit were maintained and amplified in TOP10

275   *E. coli* strain prior to submission to Addgene.

276   *C. reinhardtii* strains[35, 46, 52, 61, 62], culture and transformation conditions are recapitulated in

277   Supplementary Table 5. They were grown in Tris-Acetate-Phosphate (TAP) medium[63]

278   supplemented with agar (1.6 % m/V), spectinomycin (100 µg/mL), paromomycin (15 µg/mL),

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279   zeocin (ThermoFisher Scientific, 10 to 15 µg/mL), 5-fluoroindole (20 µM) or rapamycin (LC

280   Laboratories, 1 µM) when required. For *NIT1* promoter characterization (Figure 4a), a

281   modified TAP medium lacking nitrogen source (TAP-N) was used instead, and was

282   supplemented with 4 mM KNO_3 (nitrate) or 7.5 mM NH_4Cl (ammonium). For NAC2

283   autotrophy test (Figure 4b), cells were grown in minimal media (HSM) for selection of

284   complemented strains. The responsiveness to B12 was assessed on plate and then in liquid.

285   Cells were grown for 15 days in HSM until 1-5 $x10^7$ cells/mL concentration prior to

286   inoculation in a 96-well plate at a concentration of 10^5 cells/mL in 200 µL of HSM. For

287   response assays (Figure 4c) thiamine (Melford Laboratories Ltd.), 4-methyl-5-(2-

288   hydroxyethyl) thiazole (HET) and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP,

289   Fluorochem UK) were added to TAP media at a final concentration of 10 µM.

290   For transformation by electroporation (see Supplementary Table 5), a TAP culture of 1-5 x

291   10^6 cells/mL was concentrated 100 times in TAP complemented with 60 mM sucrose or the

292   MAX Efficiency Transformation reagent for Algae (ThermoFisher scientific) and 25-250 µL

293   were incubated with 80-300 ng of DNA for 10-30 min on ice in a 0.4 cm gapped cuvette

294   (BioRad) prior to electroporation (BioRad Gene Pulser Xcell). The cells were then left to

295   recover in TAP complemented with 40-60 mM sucrose for 16 h under appropriate light and

296   shaking conditions (typically 50 µmol photon $m^{-2} s^{-1}$ at 100 rpm) prior to plating on TAP-agar

297   plates with adapted antibiotics. Transformation by glass-beads method followed previously

298   published protocols[7, 64]. Briefly, after growth in TAP until 5 x 10^6 cells/mL, cells were

299   concentrated 30 times and 5 x 10^7 cells were mixed with DNA using glass beads. After 2-fold

300   dilution with TAP, 2.5 x 10^7 cells were spread onto TAP agar plates containing 100 µg ml^{-1}

301   spectinomycin. Plates were incubated for 16 h in the dark prior to light exposition (30 µmol

302   photon.$m^{-2}.s^{-1}$). When colony counting was performed (Figure 3a), it was 8 days after the

13

303   beginning of light. In both cases, the transformation protocol leads to insertion of a linear

304   DNA in a random location within the nuclear genome.

305

306   **Design**. All *in silico* sequence designs and analysis were performed with Serial Cloner,

307   Benchling, SnapGene, ApE or Genome Compiler. For exogenous parts, reverse translation

308   was performed with Serial Cloner using *C. reinhardtii* nuclear genome codon frequency

309   (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055).

310   amiRNAs can be generated using DNA parts pCM0-068 and pCM0-069. Both are derived

311   from the endogenous pre-miR1157[65], but differ in the way in which the amiRNA specific

312   sequence is introduced. pCM0-069/pCMM-20 is analogous to pChlamyRNA3[65], and a dsDNA

313   fragment containing the amiRNA/loop/amiRNA* sequence is introduced into a *Spe*I site

314   inside the miRNA precursor sequence. pCM0-068 presents two divergently oriented *Bpi*I

315   sites, allowing the cloning of the dsDNA fragment by Golden Gate. In this last case, the

316   dsDNA fragment is formed by the annealing of two oligos with the following sequence: 1)

317   sense   oligo   (5'   AGTA-(MIRNA*SEQ)-

318   TCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTA-(MIRNA SEQ)-T 3'), 2) anti-sense

319   oligo   (5'CAGT-A-(rev   com   MIRNA   SEQ)-

320   TAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGA-(rev com MIRNA*SEQ) 3'). There

321   are   online   tools   that   help   with   the   design   of   the   amiRNA   sequence

322   (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi).

323

324   **Parts repository.** All sequences listed in Supplementary Table 2 were deposited in Addgene.

325   Physical distribution of the DNA is performed through Addgene. We invite the community to

14

326   share their future parts through Addgene and/or with our consortium (contact M. Schroda)

327   which will make them available to the community.

328

329   **Parts cloning.** All PCR reactions were performed using the Phusion DNA polymerase, KOD

330   Xtreme Hot Start DNA polymerase (Merck) or Q5 DNA polymerase purchased from New

331   England Biolabs (NEB) following the manufacturer's instructions adapted to GC-rich DNA,

332   typically duration of hybridization and polymerization was doubled and/or GC enhancer

333   solution was used. Molecular biology kits were purchased from Macherey-Nagel, peqLab,

334   NEB or QIAgen (gel extraction and miniprep kits). Primers were produced by Eurofins

335   Genomics or Sigma-Aldrich while synthesized parts were obtained from Genecust, DC

336   Biosciences, IDTDNA or Sigma-Aldrich.

337

338   **MoClo Assembly Conditions.** All Restriction/ligation reactions were performed using *BbsI* or

339   *BbsI*-HF (*BpiI* is an isoschizomer) or *BsaI*-HF (NEB or ThermoFisher) together with T4 ligase

340   (NEB) in a medium containing the NEB CutSmart buffer and 1 mM ATP (with stock of 10 mM

341   solubilized in 0.1 M Tris-HCl, pH 7.9). Typical ratio between destination plasmid (100 fmol)

342   and entry plasmid/parts was 1:2. To facilitate handling of the kit for end-users, we provide

343   detailed protocols and reaction mix calculators for each type of assembly: level 0 for parts

344   (Supplementary Table 6), level 1 for modules (Supplementary Table 7) and level M for

345   devices (Supplementary Table 8).

346

347   **Quality Control of generated DNAs.** All plasmids were controlled by differential restriction.

348   In addition, all level 0 plasmids were sequenced with specific primers. Sequencing was

15

349   performed by Eurofins Genomics, Source BioSciences UK, Seqlab, Macrogen, Microsynth,

350   GATC Biotech or Core Facility (CeBiTec, Bielefeld University).

351

352   **NanoLuc activity determination.** Reagents were purchased from Promega (ref. N1110) and

353   activity was determined as previously described[39]. For screening, *C. reinhardtii* colonies were

354   transferred into a 96-well plate containing 100 µL of TAP in each well. After gentle

355   resuspension, 50 µL was transferred into a solid white 96-well plate to which 50 µL of Nano-

356   Glo substrate diluted in the provided buffer (2% V/V) was added and gently mixed by

357   pipetting. Luminescence was measured at 460 nm with a CLARIOstar plate reader (BMG

358   Labtech). For promoter/terminator combination assessment experiment (Figure 3c), all *C.*

359   *reinhardtii* colonies from a transformation event were pooled and resuspended in TE buffer

360   (50 mM Tris-HCl pH 7.9, 1 mM EDTA) complemented with anti-protease (1 tablet per 50 mL,

361   Sigma-Aldrich: S8830). The cells were lysed by vortexing 10 seconds twice in the presence of

362   glass beads (about 1:5 ratio beads/cells V/V) prior to two centrifugations (20000 g for 10 min

363   at 4°C) to clarify the supernatant. The protein concentration was then determined using

364   Bradford reagent with a Bovine Serum Albumin standard curve and the concentration was

365   standardized to 0.5 g/L. The activity was determined in a 96-well plate in a final volume of

366   50 µL (1:1 with nano-Glo resuspended in provided lysis buffer) per well. NanoLuc activity was

367   determined on 6 different increasing protein quantities (0.1 to 2.5 µg) for each assay,

368   allowing to assess linearity of the signal.

369

370   **Absorbance measurement of cultures growing in microtiter plates**

371   Growth in microtiter plates was determined by measuring the optical density of each well at

372   730 nm. Microtiter plates containing 180-200 µL culture were incubated under constant light

16

373 (125 µmol photon.m^{-2}.s^{-1}) at 25°C and 40 rpm orbital shaking. For density determination,

374 cultures were resuspended by pipetting and 100 µL of cell suspension was transferred to a

375 new microtiter plate containing 50 µL TAP 0.03% Tween-20. Optical density of each well was

376 determined at 730 nm in a CLARIOstar plate reader (BMG Labtech). Plates were shaken for

377 6-10 sec at 600 rpm before measurement.

378

379 **RNA extraction and miRNA-mediated cleavage mapping**

380 RNA isolation was carried out as previously described[49] (a detailed protocol can be found at

381 http://www.plantsci.cam.ac.uk/research/davidbaulcombe/methods/downloads/smallrna.pd

382 f/view), with the following modifications: Cells were centrifuged and resuspended in 0.25 mL

383 of water and mixed with 0.25 mL Lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 50 mM

384 EDTA, 2% SDS, 1 mg/mL proteinase K). Lysis buffer was incubated at 50°C for 5 min prior

385 mixing with cells. Cell suspension was then incubated at 25°C for 20 min. Finally, 2 mL of

386 PureZol (Biorad) was added and samples were snap-frozen. RNA quality was assessed in gel

387 and quantified in Nanodrop (ThermoFisher scientific).

388 miRNA cleavage site determination was performed as previously described[66]. Briefly, 10 µg

389 of total RNA was ligated with an RNA oligo (5' CGACUGGAGCACGAGGACACUGACAUGGAC

390 UGAAGGAGUAGAAA 3') using T4 RNA ligase for 1 h at 37°C. RNA was extracted with

391 phenol:chloform and precipitated with ethanol and sodium acetate. The precipitated RNA

392 was retrotranscribed into cDNA by SuperScript IV reverse transcriptase (ThermoFisher

393 scientific), using random hexamers and following manufacturer's recommendations. Two µL

394 of the cDNA was used as template of a PCR using primers FJN456 (5'-

395 CGACTGGAGCACGAGGACACTGA) and FJN495 (5'- TGGGGTAGGGGTGGGGGCCAG). Two µL of

396 this PCR was used as template of a second PCR with primers FJN457 (5'-

17

397　　GGACACTGACATGGACTGAAGGAGTA) and FJN496 (5'- TGACCCAGTCGCGGATGGCCT). PCR

398　　was resolved in a 2% agarose gel and the specific band was isolated from the gel and cloned

399　　into pGEM-T easy (Promega) for sequencing.

400

401　　**Immuno-blotting.** Chlamydomonas cells expressing FKB12 fusion proteins from liquid

402　　cultures were collected by centrifugation 4000 g for 5 min at room temperature (RT),

403　　washed in 50 mM Tris-HCl pH 7.5, and resuspended in a minimal volume of the same

404　　solution. Cells were lysed by two cycles of slow freezing to -80°C followed by thawing at RT.

405　　The soluble cell extract was separated from the insoluble fraction by centrifugation (15000 g

406　　for 20 min at 4°C). Total protein extracts (15 μg) were then subjected to 15% SDS-PAGE.

407　　mCherry-expressing cells were harvested at 3500 rpm for 2 min (4 °C) and resuspended in

408　　60 μL of DTT-carbonate buffer (0.1 M DTT, 0.1 M Na_2CO_3). After freezing at -20 °C and

409　　thawing, 55 μL of SDS-Sucrose buffer were added (5 % SDS, 30 % sucrose). Samples were

410　　then boiled for 45 s at 95 °C, followed by 2 min incubation on ice and 13000 g centrifugation

411　　for 2 min at RT. Protein extracts corresponding to 2 μg of Chlorophyll were then separated

412　　using 12% SDS-PAGE.

413　　For immunoblot analyses, proteins were then transferred to nitrocellulose membranes (Bio-

414　　Rad, 162-0115 or Amersham Protran). After blocking with 3 to 5% low-fat Milk in PBS for 1 h

415　　at RT, membranes were incubated with primary antibody in 5% low-fat Milk in PBS for 16 h

416　　at 4°C. After 4 washes in PBS - 0.1% tween-20 (TPBS), the membranes were incubated with

417　　secondary antibody in 5% low-fat Milk in PBS for 1 h at RT, and subsequently washed 4 time

418　　in TPBS prior to chemi-luminescence revelation using ECL. Primary antibodies used were

419　　anti-FKBP12 [52] (1/5000 dilution; secondary was anti-rabbit 1/10000), anti-FLAG (Sigma-

420　　Aldrich F1804, 1/5000 dilution; secondary was anti-mouse 1/5000), anti-STREP (IBA, Catalog

18

421   N. 2-1509-001, 1/5000 dilution; conjugated to HRP), anti-cMYC (Sigma-Aldrich M4439,

422   1/2500 dilution; secondary was anti-mouse 1/5000), anti-HA (Sigma-Aldrich H9658, 1/5000

423   dilution; secondary was anti-mouse 1/2500) and anti-PRPL1[67]. For mCherry serum, rabbits

424   were immunized against purified full-length mCherry protein containing an N-terminal His$_6$-

425   tag.

426

427   **Microscopy.** For mCherry experiments (Figure 6b-e), images were taken at 100x

428   magnification with a BX53F microscope (Olympus). Fluorescence images for the detection of

429   mCherry were taken using a TRITC filter. For other fluorescent proteins (Figure 6f-h),

430   microscopy was performed as previously described [7, 68].

431

432   **Accession numbers.** All parts accession numbers and the corresponding references are listed

433   in Supplementary Table 2.

434

435   **ASSOCIATED CONTENT**

436   **Supporting Information.**

437   Supplementary Figure 1 - MoClo assembly workflow reflecting the abstraction hierarchy

438   Supplementary Figure 2 - Variability of Nanoluc expression in pCMM-1 transformants.

439   Supplementary Figure 3 - Control of gene expression, complementary data.

440   Supplementary Table 1 - list of all unique parts of the Chlamy MoClo kit

441   Supplementary Table 2 - list of all parts of the Chlamy MoClo kit: level 0 plasmids

442   Supplementary Table 3 - list of all modules used for the Chlamy MoClo kit validation: level 1

443   plasmids

444   Supplementary Table 4 - list of all modules used for the Chlamy MoClo kit validation: level M

445   plasmids

446   Supplementary Table 5 - list of Chlamydomonas reinhardtii strains and associated

447   transformations

19

448   Supplementary Table 6 - level 0 ligation file: protocol and reaction mix calculator to clone
449   parts.

450   Supplementary Table 7 - level 1 ligation file: protocol and reaction mix calculator to
451   assemble modules.

452   Supplementary Table 8 - level M ligation file: protocol and reaction mix calculator to assemble
453   devices.

454

455   **Abbreviation.**

456   MoClo: Modular Cloning, TU: Transcriptional Unit, RBCS2: Ribulose Bisphosphate

457   Carboxylase oxygenase Small subunit 2,HSP70: Heat Shock Protein 70, AR: HSP70A/RBCS2,

458   TUB2: Tubulin 2, PSAD: Photosystem I reaction center subunit II, HET: 4-methyl-5-(2-

459   hydroxyethyl) thiazole, HMP: 4-amino-5-hydroxymethyl-2-methylpyrimidine, amiRNA:

460   artificial micro RNA, TAP: Tris Acetate Phosphate

461

462   **Author Information.**

463   K. Vavitsas current address is: Australian Institute for Bioengineering and Nanotechnology

464   (AIBN), The University of Queensland, Australia

465

466   **Author Contribution.**

467   SDL, AGS, MS, PEJ, OK, JLC and GP created the consortium that led this study.

468   PC, FJN, FW, PM, DCB, GP, JLC, OK, PEJ, MS, AGS and SDL designed the study and wrote the

469   manuscript.

470   PC, FJN, FW, PM, KB, KJL, MEPP, PA, AGR, SSG, JN, BS, JT, RT, LW, KV, TB, KS, MC, FdC, AD,

471   MdM, JH, WH, CHM designed parts, modules and devices, performed the experiments,

472   and/or analyzed data.

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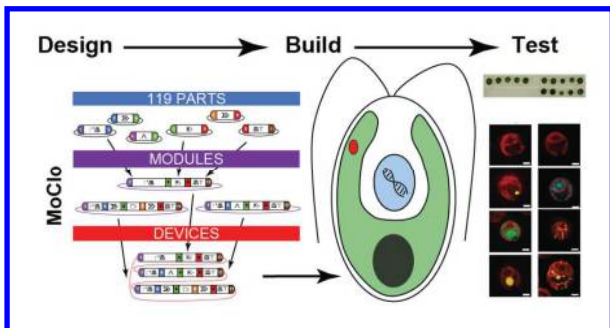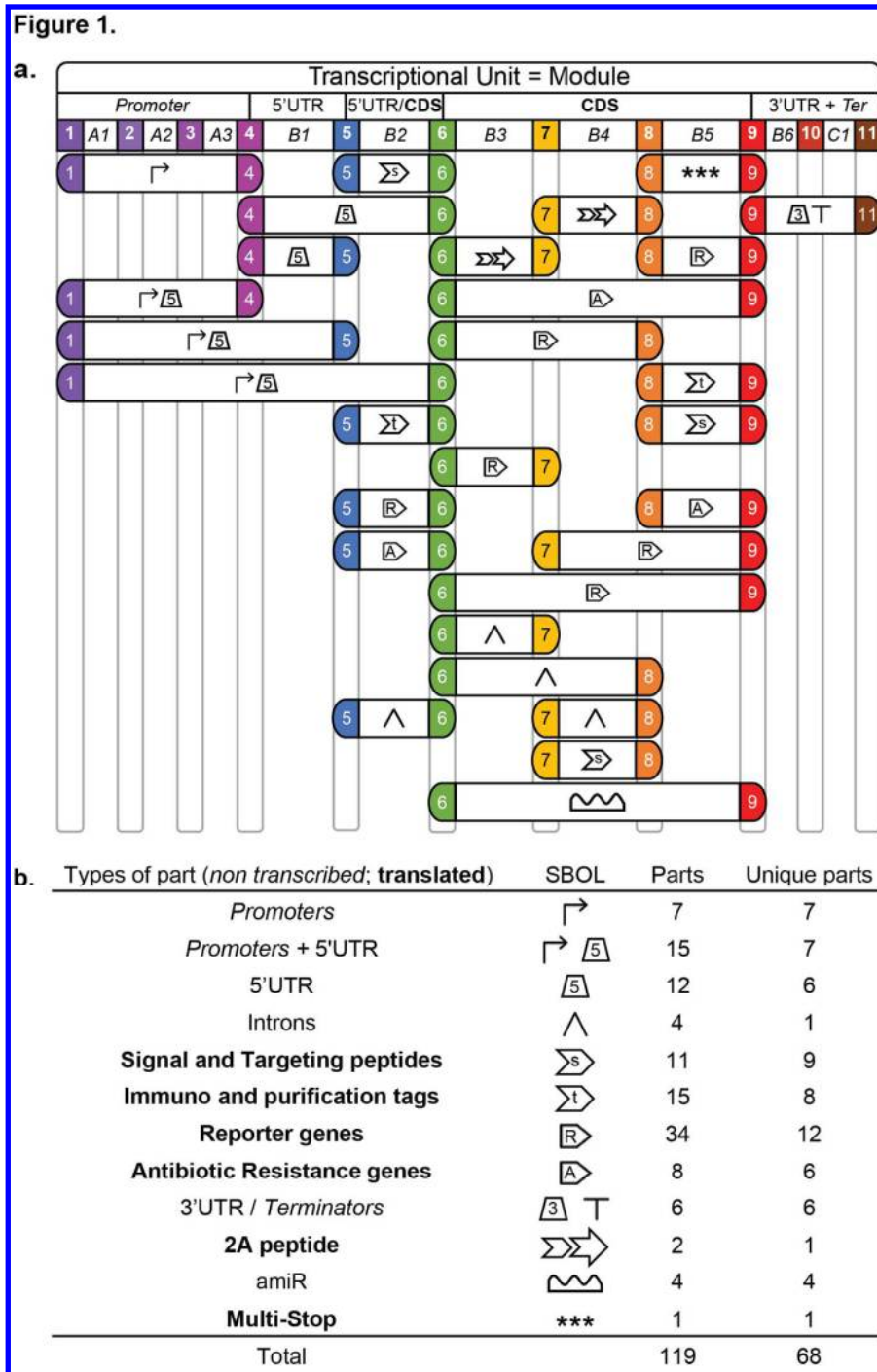
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702   **Graphical Abstract**

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Figure 1. Overview of the Chlamydomonas MoClo toolkit.

(**a**) Type and position of parts used following the Plant MoClo Syntax[30]. Symbols correspond to the SBOL2.0 visual[69] representation described in **b**. Each of the 11 fusion sites defining a part position is represented with a color and a number. Positions presented are representative of the whole set of each part type. Parts in italicized letters are non-transcribed, parts in regular letters are transcribed and parts in bold letters are transcribed and translated.

(**b**) Table summarizing unique and total gene parts available. The SBOL2.0 symbols are indicated for each type. When the SBOL2.0 standard was not existing for a part type, the symbol proposed before[28] was used, or defined here.

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Figure 2. List of parts in function of their type and assembly position.

(a) Plant MoClo syntax[30] indicating the color code for fusion sites used in this figure.

(b) All parts in the Chlamydomonas MoClo kit are classified primarily by their function, indicated by SBOL2.0 visual code[69] as in Fig. 1 (from left to right: promoters, promoter+5'UTR, 5'UTR, introns, antibiotic resistance genes, reporter genes, artificial microRNA, immunological and purification tags, 2A peptide, and 3'UTR+terminators). Colored stripes on the left and right sides of each box represent the fusion sites in 5' and 3' of the position, respectively, and follow the color code on top. AR and A'R stand for HSP70A/RBCS2 and HSP70A467/RBCS2, respectively[36]. A star (*) indicates that the part contains extra restriction sites as in pOpt vectors[68] while the same part unmarked does not. An (i) indicates the presence of an intron within the part (*cf.* Supplementary Table 2). For amiRNA (amiR) backbones, *b* and *s* mean that *Bpi*I and *Spe*I site are within the backbone for amiR cloning, respectively, while M and sc mean that the target amiR sequence for *MAA7* and the control scrambled sequence were introduced into the miR1157 backbone, respectively (cf. Fig. 4). mSTOP stands for multi-STOP.

Figure 3. Constitutive promoters and reporter genes.

(**a**) Average number of spectinomycin resistant colonies after transformation of UVM4 cells (mean ± SD, N=11) for the three modules (pCM1-3, where pCM stands for plasmid Chlamydomonas Moclo). Representative transformation plates are shown.

(**b**) Linearity of NanoLuc activity as a function of cell number. NanoLuc activity for 4 independent clones transformed with the pCMM-1 device (pCMM-1.X), one non-expressing clone (NC) and the recipient strain (CC-4425 noted as control) are presented (N=3, mean ± SEM). Linear regression and correlation coefficient (R^2) are shown. The NC and control are shown in the inset on a different scale.

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740   (**c**) Average NanoLuc activity of D66 (CC-4425) cells transformed with 4 devices (pCMM-1 to 4)

741   harboring promoter/terminator combinations to drive NanoLuc expression coupled to a

742   paromomycin resistance module (represented as P^R, left, Supplementary Fig. 2). Luminescence levels

743   are represented as mean ± SEM (average of a total of more than 400 clones from 3 biological

744   replicates). The negative and positive controls are the recipient strain and the pCMM-1.9 strain

745   (shown in **b**), respectively.

746   **a,c** *p<0.05; **p< 0.01 assessed by Student's t-test, SBOL2.0[69] visual of module designs are shown

747   above the devices.
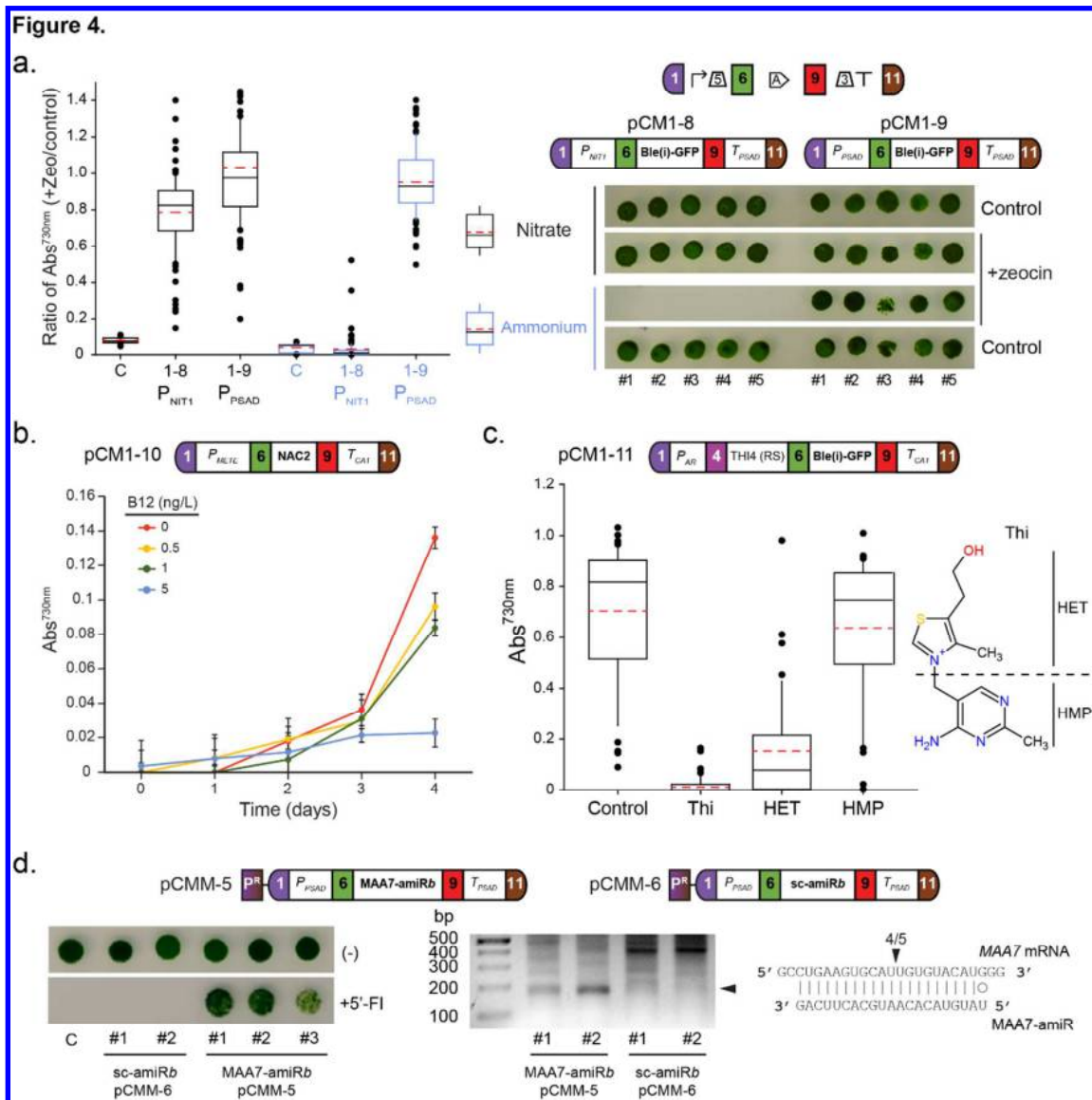
Figure 4. Control of gene expression.

(**a**) Control of gene expression by the nitrogen source. Zeocin resistant colonies (conferred by *Ble(i)-GFP*) selected after transformation of CC-1690 cells with each of the two represented modules ("1-8" for pCM1-8 and "1-9" for pCM1-9) were grown in TAP-nitrogen ± zeocin (15 µg/mL) supplemented with either 7.5 mM $(NH_4)Cl$ (ammonium, blue) or 4 mM KNO_3 (nitrate, black) and their growth was followed (Absorbance at 730 nm). The plot shows the ratio between the growth in the presence and absence of zeocin (C is the non-transformed CC-1690 strain). The right panel shows cells grown in similar conditions but on solid media. Results presented (N=16 for control CC-1690 and N=86 for each other conditions) correspond to one out of three independent transformations (for the other two, see Supplementary Fig. 3).

(**b**) Control of gene expression by vitamin B_{12}. Conditional complementation of *nac2-26* cells with the pCM1-10 module expressing NAC2 under P_{METE} control. Complemented strains were selected for photoautotrophic growth on solid minimal medium and the cells were grown in liquid minimal medium supplemented with the indicated amount of vitamin B_{12}. Data are mean ± SD (N=3).

(**c**) Control of gene expression by vitamin B_1. Average growth (absorbance at 730 nm after 7 days of growth, N=40) of UVM4 cells transformed with the pCM1-11 module designed to express constitutively *Ble(i)-GFP* transcripts containing the *THI4* riboswitch in the 5'UTR. After culture in TAP, the cells were transferred to TAP+zeocin (10 µg/mL) supplemented with 10 µM thiamine (Thi), 10 µM 4-methyl-5-(2-hydroxyethyl) thiazole

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766   (HET) or 10 µM 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) or not (control). The chemical structure
767   of Thi is represented on the right and the HET and HMP moieties are indicated (See also Supplementary Fig. 3).
768   (**d**) Targeted gene knockdown with artificial miRNA. Paromomycin resistant cells selected after transformation
769   of CC-1690 cells by each of the two represented devices (pCMM-5 and pCMM-6), carrying an amiRNA cloned
770   with *Bpi*I and directed against *MAA7* (MAA7-amiR*b*) or a random sequence ('scrambled': sc-amiR*b*), were
771   grown in the absence (denoted (-)) or presence of 5'-fluoroindole (+5'-FI) (left panel). C indicates non-
772   transformed cells. Clones resistant to 5'FI were analyzed by a modified 5'-RACE assay. A specific 173 bp PCR
773   band (black arrowhead) was amplified only from the 5'-FI resistant transformants and not from ones expressing
774   the amiRNA with scrambled sequence (middle panel and Supplementary Fig. 3). Sequencing revealed that the
775   most frequent cleavage occurred at positions opposed to positions 10 and 11 of the amiRNA (right panel, black
776   arrowhead). P^R represents the paromomycin resistance module (pCM1-27, Supplementary Fig. 2a).
777   **a, c** The box and whisker plots show the 10th (lower whisker), 25th (base of box), 75th (top of box) and 90th (top
778   whisker) percentiles. The line within the box is the median, the dashed red line is the mean. Outliers are
779   plotted as individual data points.
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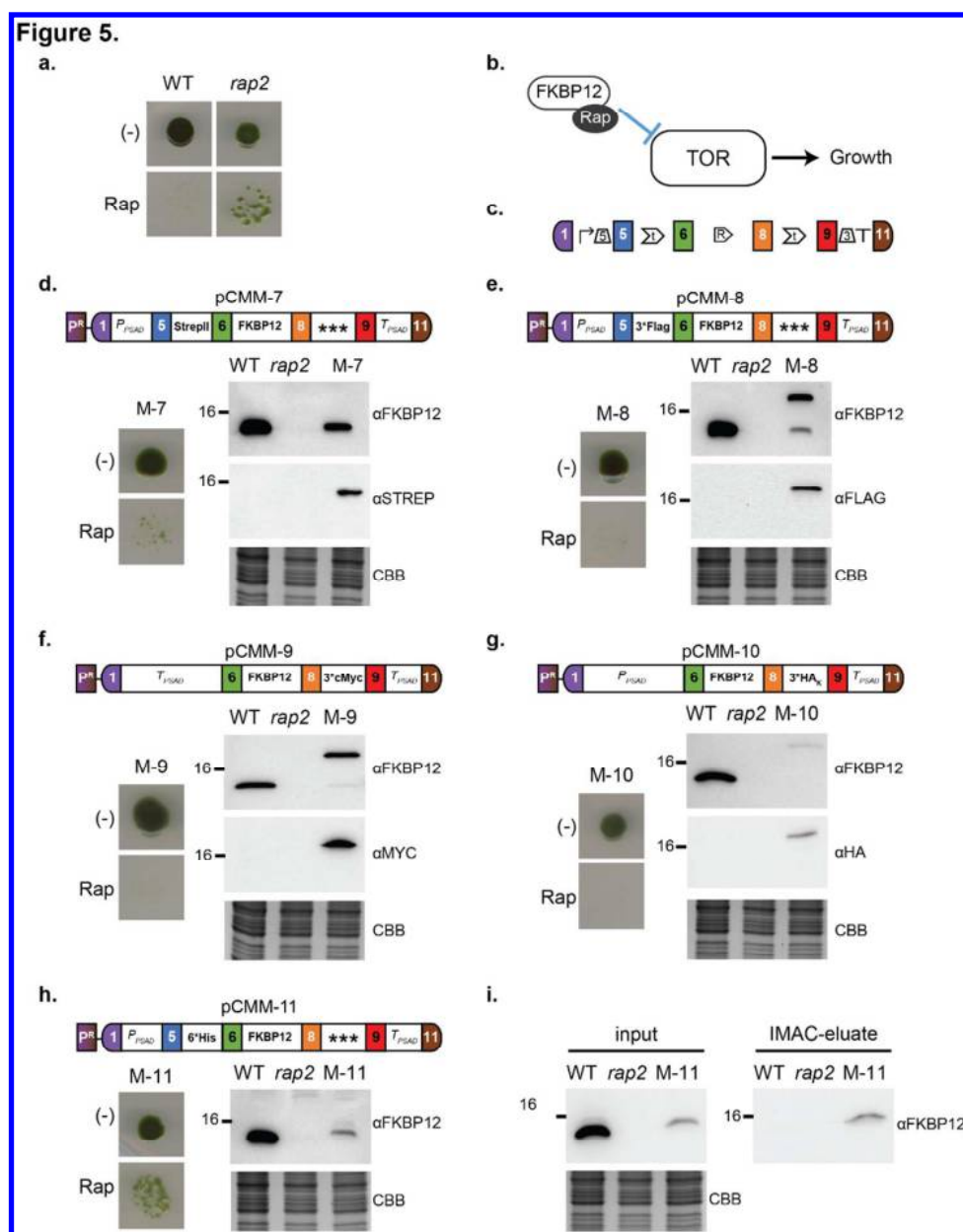
Figure 5. Design, build and test of five fusion tags

(**a**) Phenotype of recipient (WT) and Δ*FKBP12* (*rap2*) strains in the presence (Rap) or absence (-) of 1 μM rapamycin.

(**b**) Molecular mechanism underlying the *rap2* phenotype. Target Of Rapamycin (TOR) is inhibited by rapamycin only in the presence of FKBP12 (mutated in *rap2*). Upon formation of the tripartite TOR/FKBP12/rapamycin complex, TOR is inhibited and growth is arrested[52].

(**c**) SBOL2.0 visual[69] of module designs for functional complementation of *rap2*.

(**d-h**) Phenotype in the presence (Rap) or absence (-) of 1 μM rapamycin and detection of tagged proteins in soluble extracts by immunoblotting with antibodies against either FKBP12 (αFKBP12) or the appropriate tag (indicated within each panel). Each device is indicated in the upper part of the panel. CBB: Coomassie Brilliant Blue staining of a duplicate gel loaded with the same samples and shown as loading control.

(**i**) Purification through ion-metal affinity chromatography (IMAC) of 6His-FKBP12 expressed from the same device as in **h**.

P^R represents the paromomycin resistance module (pCM1-27, Supplementary Fig. 2a). *rap2* cells transformed with pCMM-X are indicated as M-X in each panel. Data are representative of 3 biological replicates.

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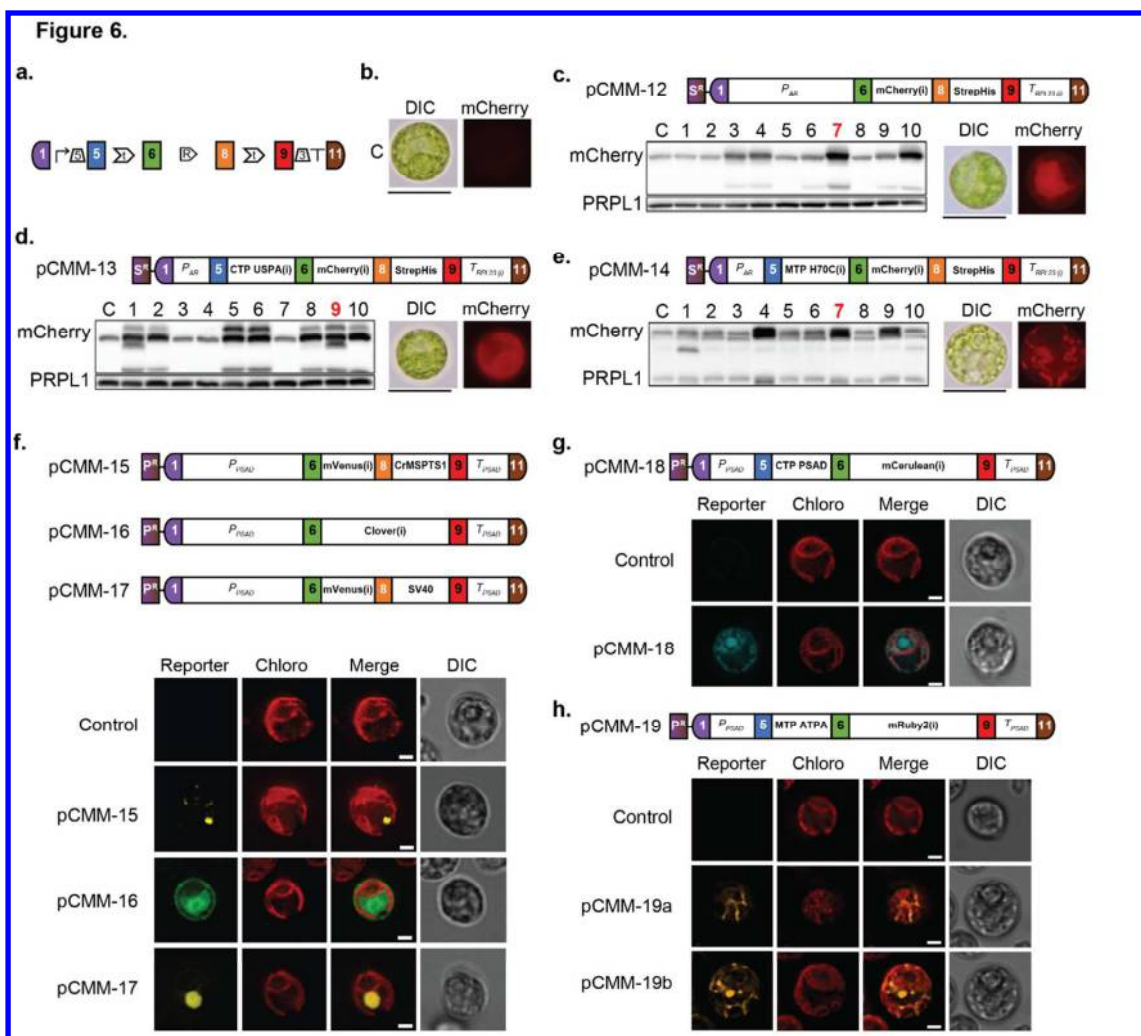
Figure 6. Targeting reporter genes to different subcellular compartments.

(**a**) SBOL2.0[69] visual syntax for modules used.
(**b**) Visible light ("DIC") and fluorescence signal ("mCherry") of the UVM4 recipient strain used as control ("C")
for panels **c-e**.
(**c-e**) mCherry targeting to the (**c**) cytosol with no transit peptide, (**d**) chloroplast with CTP USPA (Chloroplast
Transit Peptide of Universal Stress Protein A) or (**e**) mitochondria with MTP H70C (Mitochondrial Transit
Peptide of HSP70C) in UVM4 cells transformed with the indicated devices (pCMM-12 to 14). In each panel, an
anti-mCherry immunoblot analysis of transformants is shown. Note that the anti-mCherry antibody cross-reacts
with a protein of similar size present in control cells (C). An anti-PRPL1 immunoblot is shown as loading control.
The transformant strain number indicated in red corresponds to the images (bars are 10 µm) presented on the
right.
(**f-h**) Fluorescent marking of (**f**) microbodies with mVenus-CrMSPTS1 (Malate Synthase PTS1-like sequence),
cytosol with Clover or the nucleus with mVenus-SV40 (Simian Virus 40 nuclear localization signal), (**g**) the
chloroplast with CTP PSAD-mCerulean (Chloroplast Transit Peptide of PSAD), (**h**) mitochondria with MTP ATPA-
mRuby2 (Mitochondrial Transit Peptide of ATPA) after transformation of UVM4 cells with the indicated devices
(pCMM-15 to 19). Images of representative transformants are grouped with the corresponding control image
(recipient strain) according to the filter used. pCMM-19a and pCMM-19b show two images taken on different
z-axis on the same cell. "Chloro" refers to chlorophyll autofluorescence. The Scale bars represent 2 µm.
S^R and P^R represent respectively modules conferring resistance to spectinomycin (S^R=pCM1-1, Fig. 3a and
Supplementary Fig. 2a) and paromomycin (P^R=pCM1-27, Supplementary Fig. 2a).