CpPosNeg: a positive-negative selection strategy allowing multiple cycles of marker-free engineering of the Chlamydomonas plastome

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Abstract

The chloroplast represents an attractive compartment for light-driven biosynthesis of recombinant products, and advanced synthetic biology tools are available for engineering the chloroplast genome (=plastome) of several algal and plant species. However, producing commercial lines will likely require several plastome manipulations, and this will present issues with respect to selectable markers: there are a limited number of markers available, these can be used only once in a serial engineering strategy, and it is undesirable to retain marker genes for antibiotic resistance in the final transplastome. To address these problems, we have designed a rapid iterative marker system for the green microalga Chlamydomonas reinhardtii that allows creation of marker-free transformants starting from wild-type strains. The system employs a dual marker encoding a fusion protein of E. coli aminoglycoside adenyltransferase (conferring spectinomycin resistance) and a variant of E. coli cytosine deaminase (conferring sensitivity to 5-fluorocytosine). Initial selection on spectinomycin allows stable transformants to be established and driven to homoplasmy. Subsequent selection on 5-fluorocytosine results in rapid loss of the dual marker through intramolecular recombination between the marker's 3'UTR and the 3'UTR of the introduced transgene(s). We demonstrate the versatility of the CpPosNeg system by serial introduction of reporter genes into the plastome.

1 Introduction

The chloroplast of plants and algae is not only the site of photosynthesis, arguably the most important biological process on the planet, but is also a major biosynthetic compartment within the cells of these photoautotrophic organisms.[1] Beyond their fundamental role in nature, chloroplasts possess many traits that make them attractive as sub-cellular platforms for industrial biotechnology. They possess a small polyploid genome (=plastome) derived from their cyanobacterial ancestor, which has retained only a hundred or so genes, many of which are highly expressed. Foreign genes can be integrated precisely into the plastome via a process of homologous recombination allowing targeting of these genes into neutral loci, thereby avoiding any position effects. Furthermore, since the chloroplast genetic system lacks any gene silencing mechanisms, high levels of expression and recombinant protein accumulation are achievable without the need to maintain selection.[2,3]

Chloroplast transformation was first achieved in 1988[4] using the unicellular green alga *Chlamydomonas reinhardtii* . Since then, this species has been used extensively to demonstrate the potential of the algal chloroplast as a chassis for synthesis of recombinant products including therapeutic proteins,[5] novel metabolites,[6] and bioactive RNAs.[7,8] An ever-growing 'chloroplast toolkit' for *C. reinhardtii* now allows routine insertion of codon-optimised transgenes into the plastome, and their high-level and regulated expression.[9] More

recently, there has been a growing emphasis on the utilisation of synthetic biology (SynBio) approaches to chloroplast engineering.[5,10–12]This has been supported by the availability of robust, well annotated genomic and transcriptomic data for the *C. reinhardtii*plastome[13,14] and the emergence of standardised DNA assembly methods for rapid and high-throughput design and construction of transgenes.[15] These enabling technologies are now facilitating progression from simple genetic engineering strategies based on one or two transgenes to the integration and effective regulation of multiple transgenes, allowing the introduction of novel metabolic pathways into the algal plastid,[16] and radical refactoring of the plastome.[17]

These ambitious engineering efforts will likely require several rounds of engineering of the same strain, either to introduce multiple transgenes for metabolic engineering, or to perform plastome rearrangements and deletions.[9] However, such advanced transplastomics is currently constrained by the paucity of different selectable markers for chloroplast transformation of *C. reinhardtii* .[18] For example, only three bacterial genes have been developed to-date as portable markers: the*aadA* cassette conferring spectinomycin resistance,[19] the *aphA6* cassette conferring kanamycin resistance,[20] and the*ptxD* cassette that allows phosphite auxotrophy.[21] Moreover, each round of engineering involves the permanent introduction of a marker into the plastome as well as the gene(s) of interest. This not only prevents the re-use of the marker in subsequent transformations of the strain, but also results in strains carrying unnecessary and undesirable bacterial genes. Commercial cultivation and utilisation of such strains (e.g. as oral vaccines[22]) is therefore associated with risks of horizontal transfer of these genes to other microorganisms.[23]

Several strategies to circumvent these issues have been developed for*C. reinhardtii* . Plastome mutants carrying defects in a gene required for photosynthesis can be used as recipient strains whereby selection is based on the restoration of phototrophy through repair of the defective gene, thereby generating a marker-free transgenic line. However, this limits transformation to a specific strain and such a selection strategy can be utilised only once.[18]Fischer et al. developed an alternative strategy for generating marker-free lines by using an *aadA* cassette that was flanked by direct repeat sequences.[24] Following integration into the plastome and selection for homoplasmy of the transformed plastome, the selective pressure is removed allowing the marker to be lost from the plastome via intramolecular recombination between the repeats. Loss of the *aadA* cassette leaves just a single copy of the repeat sequence as a DNA 'scar' at the site of plastome integration, and the cassette can be reused in further rounds of transformation. A similar strategy has been developed for higher plant chloroplasts, with the issue of the unwanted scar being avoided by creating a direct repeat using endogenous sequence adjacent to the integration site, rather than two copies of an exogenous element.[25]

The main limitation of the *aadA* recycling method is that the direct repeat needs to be of a significant size (0.42 kb or larger) in order to achieve sufficient rates of intramolecular recombination in the absence of active selection. Even so, complete loss of the marker can still involve time consuming cycles of replating on selective media and extensive screening[24–26]. The use of larger direct repeat sequences can increase the rate of intramolecular recombination[24] but poses several issues. If the direct repeats are incorporated in the endogenous regulatory elements used to drive expression of the marker, this may result in unwanted recombination with the original copy of this element elsewhere of the plastome, yielding a persistent heteroplasmic state due to deletion of essential genes[27] or unwanted deletion of non-essential genes.[28] Alternatively, if the direct repeat is external to the marker, then a large tract of foreign DNA is left as a scar, potentially perturbing plastome function.[24]

To address these issues, we have developed the so-called CpPosNeg system for scarless recycling of the marker in the *C. reinhardtii*chloroplast. This system uses a dual selectable marker encoding a CodA–AadA fusion protein that confers both positive and negative selection. The marker is linked to a transgene such that both share the same 3' untranslated region (3'UTR) thereby creating a direct repeat. Introduction of the construct into the plastome involves a two-step process with transformants initially selected for spectinomycin resistance conferred by the AadA moiety. Recombination between the repeats would then be promoted by a strong negative selection on 5-fluorocytosine with CodA converting it to the toxic product, 5-fluorouracil.[29]

We demonstrate the utility of the method by creating two marker-free transgenic lines with a luciferase gene inserted into different loci within the WT plastome. To demonstrate the iterative capability of the

system, a second round of CpPosNeg is used to introduce an additional reporter gene into the plastome. Since both *aadA* and *codA*have also been developed as selectable markers for the tobacco chloroplast,[30,31] the CpPosNeg system could be easily adapted for engineering of plant plastomes.

2 Materials and Methods

Strains and culture conditions

C. reinhardtii strain CC-1690 was acquired from The Chlamydomonas Resource Center (University of Minnesota) and used as the parental cell line for all transformants with the exception of the CrCD transformant which was described previously.[32] Strains were maintained on 1.5% agar plates containing TAP medium[33] at 25°C and a light intensity of ˜50 µE. Where appropriate, spectinomycin (Spc: Sigma-Aldrich; S4014) and 5-fluorocytosine (5-FC: Sigma-Aldrich; F7129) were added to agar plates at a concentration of 300 µg mL^{-1} and 5 mg mL^{-1}, respectively. 20 mL liquid cultures were prepared from freshly grown agar plates (2-3 days) in 50 mL Erlenmeyer flasks and incubated at 25°C, ˜50 µE with shaking at 120 rpm.

For growth tests on plates ('spot tests'), liquid cultures were grown to mid-log phase (˜ 3 x 10^6) before being normalised by optical density at 750 nm to the lowest measured sample. Dilutions were then prepared at 1:10 and 1:100 in TAP medium and 5 µL of each dilution spotted onto TAP agar plates supplemented with either Spc or 5-FC. Plates were incubated for 1–2 weeks to allow spots to develop.

Plasmid construction

All plasmids were constructed using Start-Stop assembly[15]. This is a level-based cloning system with basic genetic elements as discrete standardised 'level 0' parts. The type IIS restriction enzyme SapI was used to assemble transcription units (level 1) from the level 0 parts and then these were combined, along with flanking arms for homologous recombination, using BsaI to create the final level 2 plasmids. Some minor modifications were made to the Start-Stop acceptor vector, which are detailed in**Supplementary File S1** along with the basic assembly strategy for the level 2 constructs. The coding sequence for the mVenus.ME variant carrying a Q69M change[34] was codon optimized for the *C. reinhardtii* chloroplast, ordered as a synthetic gene termed *mVenCP* (GeneArt; Thermo Fisher Scientific), and cloned into a level 0 acceptor vector. See **Supplementary File S2** for GenBank format vector maps of all level 2 constructs assembled in this study.

Transformation of *C. reinhardtii*

Plasmids were delivered to the *C. reinhardtii* chloroplast using microprojectile bombardment [35] with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, USA). Cells were grown to a cell density of 2 x 10^6 cells/mL (early mid-log phase), harvested by centrifugation, and plated on 1.5% TAP agar plates at concentration of 1 x 10^8cells/plate (bombardment plates hereafter). Gold DNAdel carrier particles (Seashell Technology) were coated with the appropriate plasmid DNA prior to bombardment of the plates. Plates were incubation in low light (˜10 µE) overnight and then the lawn of cells harvested in 1 mL of TAP medium and plated on 1.5% TAP+Spc plates at a concentration of 5 x 10^7 cells/plate. Plates were incubated in ˜50 µE light at 25°C until colonies appeared and were large enough to re-streak (˜ 2 weeks). Several colonies for each strain were re-streaked to single colonies on TAP+Spc plates several times to obtain homo-transplastomic lines. To induce loop out of the selection cassette, strains were re-streaked to single colonies twice on TAP+5-FC plates. Integration of foreign DNA and homoplasmy of the plastome was checked by PCR analysis of genomic DNA extracted from single colonies using the Chelex method.[36] Primers used in the analysis are detailed in **Supplementary Table 1** .

Luminescence and fluorescence assays

Luminescence and fluorescence assays were performed on mid-log grown cells, normalised to the lowest recorded optical density at 750 nm, in 96-well microplate format using a FLUOstar Omega Microplate Reader (BMG Labtech, Buckinghamshire, UK). Absorbance measurements (OD_{750}) were performed in Greiner CELLSTAR round bottom clear well microplates (Sigma-Aldrich; M9311), fluorescence measurements in

black Greiner microplates (Greiner Bio-One; 655900), and luminescence measurements in white Eppendorf microplates (Thermo Fisher Scientific; 15294516).

Luminescence analysis of *LucCP* expression was performed using the Steady-Glo Luciferase Assay System (Promega UK Ltd, Southampton, UK). 100 µL of normalized cells were mixed with 100 µL of the Steady-Glo assay reagent in triplicate. After a 5 min incubation period, the luminescence signal was measured over the entire visible light range to maximize the signal to noise ratio. Luminescence was measured and expressed as relative luminescence units (RLU). The average RLU measurement for each sample was expressed as a 'relative luminescence' (RLU/OD_{750}). All samples were spaced one well apart in the microplate to prevent any bleed through of luminescence signal from one well to another.

Fluorescence analysis of *mVenCP* expression was performed using 200 µL of normalized cells**.** For each set of measurements**,** samples were loaded into a black microplate in triplicate along with a blank solution containing 200 µL of TAP medium. Fluorescence was detected with the appropriate filter set for *mVenus* (505 nm excitation, 540 nm emission) and expressed as relative fluorescence units (RFU). The averaged blank RFU measurement was subtracted from all samples to give the final value expressed as 'relative fluorescence' (RFU/OD_{750}).

3 Results

3.1 The CpPosNeg marker strategy

The CpPosNeg marker-recycling strategy is divided into two recombination events, which we call R1 and R2 (**Figure 1**), leading to the final unmarked strain. Initially, integration of the CpPosNeg plasmid into the *C. reinhardtii* plastome occurs via intermolecular recombination between the ˜1000 bp left and right homology arms (LHA and RHA) of the plasmid and the corresponding regions of the plastome. Selection on spectinomycin (Spc) allows generation of an insertional cell line containing both the GOI cassette and the positive/negative selection cassette encoding the CodA-AadA fusion protein. Cell lines are serially re-streaked on Spc to eliminate any WT copies of the plastome. Depending on growth regimes, the chloroplast has on average ˜ 83 copies of the plastome,[13] and all copies within the chloroplast must contain the R1 DNA in order to proceed with the strategy and avoid reversion to the WT genotype following removal of the Spc selection pressure. In the second recombination step, cells are treated with 5-FC thereby imposing a negative selection pressure for retention of the CodA activity. This selects for loss of the dual marker cassette from the plastome via intramolecular recombination between the two copies of the *rbcL* 3' UTR element linked to the marker and the GOI, respectively.

3.2 Development of a *codA-aadA* dual marker through translational fusion

Whilst both markers have individually been shown to be functional in the *C. reinhardtii* chloroplast,[19,32] and AadA has been shown to retain functionality when synthesised as a C-terminal fusion to endogenous chloroplast proteins,[37] the creation of a dual marker conferring both Spc resistance and 5-FC sensitivity has not been demonstrated previously. We therefore created two initial plasmid constructs in which *codA* and *aadA* were fused together, either at the transcriptional or the translational level (**Figure 2**). In plasmid pC-A the coding sequences are linked via a flexible linker sequence (encoding GGSGGGSG[38]) to create a single CodA-AadA fusion protein. In pC-IEE-A the two coding sequences are transcriptionally linked as an biscistronic operon via an intercistronic expression element (IEE) derived from the endogenous *tscA –chlN* intergenic region.[39]For these initial constructs, direct repeat elements were not included so that the marker genes would remained stably integrated in the plastome.

Homoplasmic transformant lines were recovered for both constructs following biolistic transformation of the WT strain (**Figure 2b**). Phenotypic tests were then carried out by spotting cultures on selective medium. For both classes of transformant, the dual functionality of the marker was confirmed by their ability to grow on Spc and inability to grow on 5-FC, in contrast to the untransformed WT strain (**Figure 2c**).

Since both arrangements of the *codA* and *aadA* coding sequence gave very similar phenotypes, it was decided to take the translational fusion forward since this avoided introducing a duplicate copy of the IEE into

the plastome, which might promote unwanted recombination between the marker and the *tscA-chlN* locus. However, since fusing CodA and AadA might compromise the efficient folding of either enzyme moiety, and hence full enzyme activity, we tested two further linkers with respect to transformation efficiency and acquired sensitivity to 5-FC. In addition to the original flexible linker GGSGGGSG, the two proteins were connected via either a rigid helix-forming linker (LAEAAAKEAAAKAAA[40]) designed to give spatial separation of the two enzymes, or the short linker ISGANGV.[38] All three constructs yielded Spc-resistant colonies following chloroplast transformation of the WT strain, but transformation efficiencies with the rigid and short linker constructs were seen to be much lower than those obtained with the flexible linker (**Figure 3b**). We concluded that the flexible linker was the most optimal for AadA activity, and it is likely that this is beneficial for Spc selection in the initial phase of transformation when only a few plastome copies carry the marker.[41] CodA activity also appeared to be higher when fused via the flexible linker since spot tests showed greater sensitivity to 5-FC when compared to transformants generated using the rigid or short linker constructs (**Figure 3d**). In light of these results, the dual marker with flexible linker (C-A.f) was selected for construction of the CpPosNeg plasmids.

3.3 Efficient creation of marker-free transplastomic lines using CpPosNeg

To validate the CpPosNeg system (**Figure 1**), plasmids were designed in which the dual marker was placed downstream of *lucCP* , a codon-optimized reporter encoding firefly luciferase.[42] For both gene cassettes, the same 3' UTR element from *rbcL* was used in order to create a 258 bp direct repeat (see: **Supplementary Figure 1)** . This size of repeat was chosen since it is significantly smaller than the 462 bp needed for high rates of intramolecular recombination in the absence of selection in the *C. reinhardtii*chloroplast,[24] but larger than the minimum size (˜210 bp) reported for such recombination to occur.[26] The dual marker was therefore predicted to be stably maintained in the intermediate transformant lines (R1) in the absence of Spc selection, but efficiently lost in the R2 lines following counter-selection on 5-FC (**Figure 1b**). Left and right homology arms of ˜1000 bp were placed upstream and downstream of the reporter and marker cassettes in order to target the genes to two different neutral insertion sites: either downstream of*psbA* [39] (plasmid pLuc1) or downstream of*psbH* [43] (plasmid pLuc2).

WT *C. reinhardtii* was transformed using pLuc1 or pLuc2 with selection based on Spc resistance conferred by the dual marker. For each transformation, six colonies were re-streaked on Spc over three generations to drive the cells to the R1 homoplasmic state (**Figure 1d**). As illustrated in **Figure 4a** , a four-primer multiplex PCR analysis of either the *psbA* locus or the *psbH* locus was employed to confirm the genotype with diagnostic band sizes for the WT, R1 and R2 loci. All six transformant lines showed the R1 genotype and appeared to be homoplasmic following three rounds of Spc selection, with no detection of the WT band. Importantly, two further rounds of replating on medium lacking Spc demonstrated that the *codA-aadA* marker DNA was stably maintained in the plastome despite being flanked by the *rbcL* direct repeat, with the PCR analysis showing maintenance of the R1 band and no appearance of the R2 band (**Figure 4c**). Conversely, two rounds of plating on 5-FC medium led to the rapid loss of the marker, with the R2 plastome appearing to be homoplasmic since only the PCR product from primers P2 and P4 was detected (**Figure 4d**). Sequencing of this PCR product confirmed the loop-out of the marker at both the *psbA*and *psbH* loci via recombination between the *rbcL* copies.

An assay of luciferase activity in the R1 and R2 lines confirmed that the introduced *lucCP* cassette was expressed and that the level of expression was not affected by the subsequent loop-out of the cassette with both pairs of R1 and R2 lines showing similar activities (**Figure 5e**). There was a small but significant difference in expression in Luc1.R2 relative to Luc1.R1 in the conditions tested (3.6%; $p = 0.03$; Student's t -test). While this could be due to the removal of the selection cassette, it is more likely an artefact due to subtle differences in the culture history and incubation conditions of the samples. There was no significant difference between expression in Luc2.R1 and Luc2.R2 ($p = 0.13$; Student'st -test). Interestingly, the targeting of *lucCP* into the plastome's large inverted repeat region downstream of *psbA*(transformant Luc1) such that two copies are present per plastome molecule rather than one as in the case of the *psbH* transformants (Luc2) gave more than twice the luciferase activity. This suggests that the activities of the

rrnS promoter and *psaA* 5'UTR elements used to drive *lucCP* expression are not limiting, and that the level of recombinant protein is directly related to copy number. This is a surprising finding given that copy number is assumed not to be a key factor in chloroplast expression[44] and that transgene expression is mainly controlled at the translational level.[45]However, we cannot rule out genomic context and the influence of upstream/downstream transcriptional units as an alternative explanation for the different expression levels.

After removal of the *codA-aadA* cassette, the R2 phenotype should be the same as the WT with respect to sensitivity to Spc and resistance to 5-FC. To confirm this, spot tests were carried out on WT, Luc1.R2 and Luc2.R2 (**Figure 5f**). C-A.f (Spc resistant; 5-FC sensitive) was included as a positive control. Luc1.R2 and Luc2.R2 showed the same phenotype as WT: full dieback on Spc and similar levels of growth in the presence or absence of 5-FC. This further confirmed that the*codA-aadA* marker had been completely lost in the R2 cell lines.

3.5 Serial transformation is achievable using CpPosNeg

To demonstrate that the CpPosNeg method could be repeated to create marker-free strains carrying multiple transgenes, we tested whether a second reporter, *mVenCP* could be introduced into the Luc2.R2 cell line that has *lucCP* at the *psbH* downstream locus. As shown in **Supplementary Figure 1** , plasmid pVen1 was designed to target*mVenCP* to the *psbA* downstream locus, again using the*codA-aadA* marker. However, to avoid the possibility of recombinational interchange between the *lucCP* and *mVenCP*cassettes due to both having the same 5' and 3' elements, the 3'UTR used to create the direct repeat was changed from the 258 bp *rbcL* UTR to an identical sized UTR from *atpB* . As before, transformants were initially selected on Spc to achieve homoplasmy at the R1 stage, and subsequently re-streaked on 5-FC to select for loss of the marker. All transformant lines (termed Luc2:Ven1) were confirmed by PCR to have reached homoplasmy at the R2 stage (**Figure 5a,b**). Phenotypic tests carried out on Luc2:Ven1 confirmed sensitivity to Spc and resistance to 5-FC following the loss of the marker (**Figure 5c**). A representative Luc2:Ven1 line was maintained on medium without selection thereafter and displayed stable expression of the*mVenCP* (**Figure 5d**) and *lucCP* (**Figure 5e**) reporters. Relative to the Luc2 parental line, there appeared to be a small reduction in the luciferase activity of Luc2:Ven1, although this was not statistically significant ($p = 0.12$; Student'st -test). These results show that the CpPosNeg strategy can be used to serially introduced multiple transgenes into the plastome with the *codA-aadA* dual marker efficiently removed at the end of each transformation cycle.

4 Discussion

The chloroplast is a key target in algal and plant biotechnology given both its central role in photosynthesis, and as the site of synthesis for primary metabolites such as fatty acids, terpenoids and tetrapyrroles.[46] The algal chloroplast, specifically that of *C. reinhardtii* , is well suited for genetic engineering and there is an increasing emphasis on the application of synthetic biology.[5,9,11,12] Many of these approaches will be reliant on the ability to perform a series of plastome edits to the same cell line. However, conventional strategies for selection of transformants largely preclude this: methods based on photosynthetic restoration are restricted to a particular mutant host and specific locus, and can only be performed once,[43] whilst portable markers for engineering WT plastomes are currently limited to just three and these also operate on a single-use basis.[18] Recycling these markers via intramolecular recombination can circumvent this issue and also generate marker-free engineered lines[17,24]. However, these strategies have so far been reliant on passive accumulation of plastome copies that have lost the marker under non-selective conditions, requiring lengthy wait times to generate homoplasmic cell lines and the use of larger intramolecular repeat sequences to increase the rates of recombination.

Our CpPosNeg system addresses both these problems by mediating efficient loss of the dual marker through active counter-selection using 5-FC using repeat elements as small as 258 bp. This allows the 3' UTR of the marker and linked transgene to be used as the direct repeat thereby avoiding introduction of any unwanted DNA scar and producing a final transgenic line containing just the transgene. This line can then be used for further rounds of engineering. Since the choice of 3'UTR has relatively little influence on transgene expression in *C. reinhardtii* chloroplasts,[47] then different endogenous or synthetic 3'UTRs could be used

beyond the two (*rbcL*and *atpB*) used in this study, thereby avoiding having multiple transgenes with the same 3'UTR in an engineered plastome. Furthermore, the minimum size of the direct repeat could probably be smaller than the 258 bp used here since intra- and inter-molecular recombination has been shown to occur in the *C. reinhardtii* chloroplast between elements as small as 216 bp and 110 bp, respectively.[26,48].

Since the CodA enzyme retains full activity when fused via a flexible linker to AadA, then it should be possible to develop additional dual systems based on CodA. This could involve fusions to other antibiotic-resistance proteins such as AphA6[20]to create alternative CpPosNeg markers, or to reporter proteins such as GFP[18] allowing rapid fluorescence sorting of individual transformed cells[49] for those that have lost this dual reporter-marker. Finally, both *aadA* and*codA* have been shown to work as selectable markers in tobacco chloroplasts,[50] as have the *rbcL* and*atpB* 3'UTRs from *C. reinhardtii* .[51]It is likely therefore that the dual marker described here could be easily adapted for efficient serial engineering of higher plant chloroplasts. CpPosNeg could also be applied to other plastome engineering strategies based on intramolecular recombination such as marker-free deletion of endogenous genes and introduction of SNPs[25,28] thereby accelerating the field of chloroplast synthetic biology.

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8 Figures and legends

Fig 1

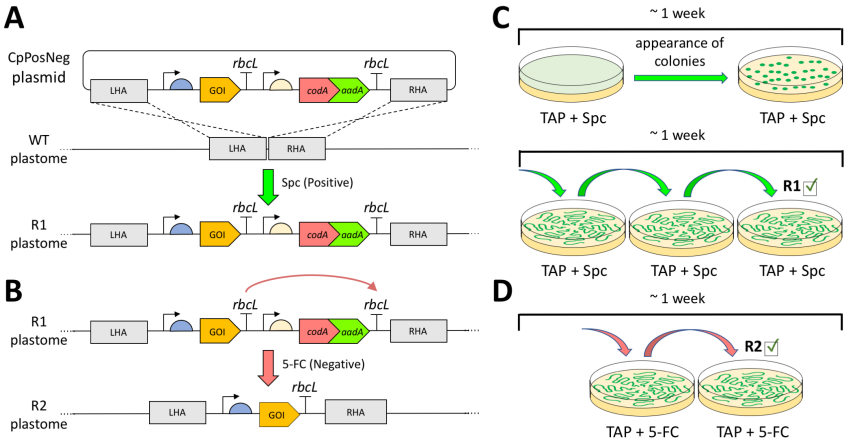

Figure 1: Marker recycling strategy. (**A, C**) Transformants are generated by integration of the CpPosNeg plasmid into a neutral site within the WT plastome via homologous recombination of left and right homology arms (LHA and RHA) and selection on Spc conferred by aadA. Initial transformants are then re-streaked on plates supplemented with Spc to eliminate all WT copies and generate the intermediate R1 plastome. (**B, D**) The R1 strains are then re-streaked on plates supplemented with 5-FC. Negative selection conferred by *codA* selects for plastome copies where the*codA-aadA* marker has been lost via intramolecular recombination of the *rbcL* direct repeat. Re-streaking on 5-FC results in the final marker-free R2 strain.

Fig 2

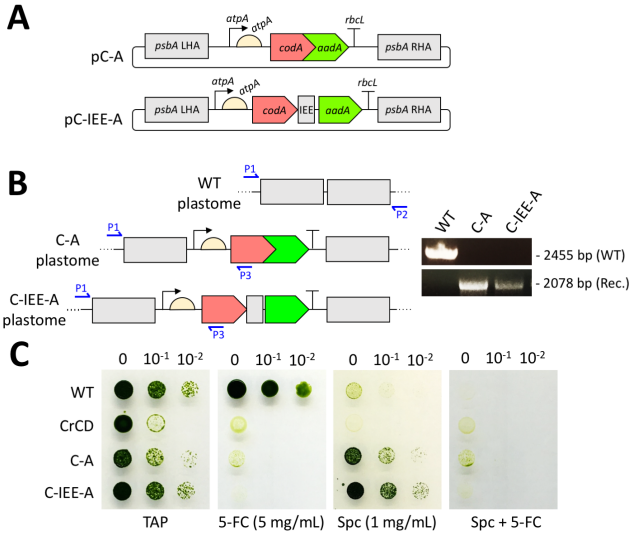

Figure 2: Validation of *codA* and *aadA*functionality as translational or transcriptional fusions. (**A**) Design of the translational and transcriptional plasmids pC-A and pC-IEE-A respectively. LHA/RHA; left and right homology arms for targeting to a neutral locus downstream of psbA. The promoter and 5'UTR elements used to drive codA/aadA expression are from atpA, and the 3'UTR from *rbcL* . IEE; *tscA-chlN* intercistronic expression element. (**B**) Three primer PCR strategy for checking homoplasmic transformants. The wild-type (WT) plastome yields a 2455 bp product with primers P1 and P2, whereas the recombinant (Rec.) plastome gives a 2078 bp product with P1 and P3. (**C**) Growth tests of mid-log cultures, and 1:10 and 1:100 dilutions, spotted on to TAP medium containing: no selective agent; Spc, 5-FC, and Spc+5-FC. Cultures

are WT, control strain CrCD expressing*codA* ,[32] representative transformant lines generated using plasmid pC-A, and pC-IEE-A.

Fig 3

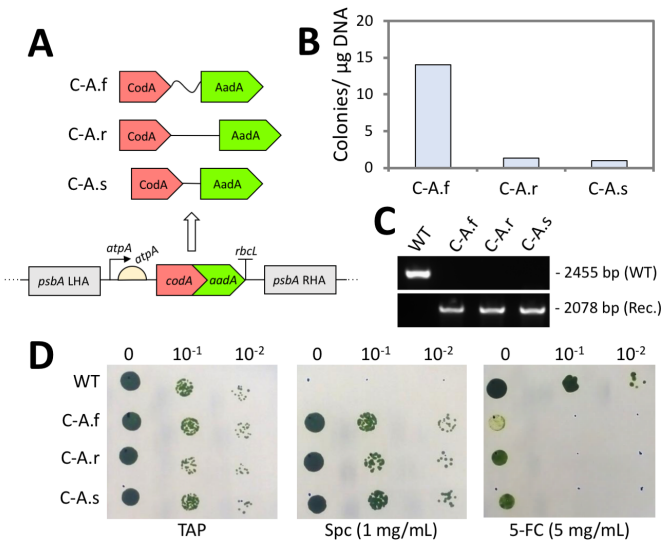

Figure 3: Effect of different linkers on activity of the AadA-CodA fusion protein. (**A**) C-A.f, C A.r and C-A.s possess a flexible, rigid and short linker, respectively. (**B**) Biolistic transformation efficiencies obtained with each construct. (**C**) PCR confirmation of plastome homoplasmy with the untransformed WT strain giving a 2455 bp product which is absent from a representative of each of the three transformant lines. Conversely, the three lines yield a 2078 bp product diagnostic of the transgenic plastome. (**D**) Spot tests of mid-log cultures and 1:10 and 1:100 dilutions on TAP plates containing: no antibiotic, Spc or 5-FC.

Fig 4

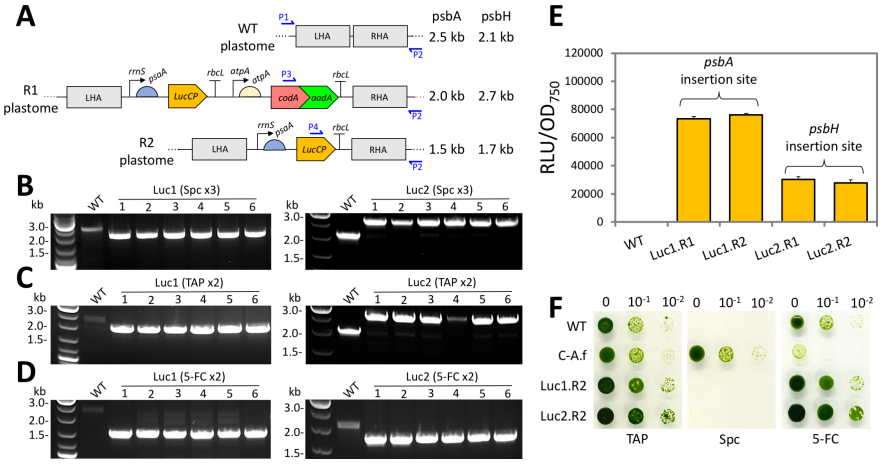

Figure 4: (**A**) Illustration of the plastome loci (i.e. downstream of *psbH* or *psbA*) prior to transgene insertion (WT), as the intermediate recombinant loci (R1), and following loop-out of the *codA-aadA* marker (R2). The locations of the primers (P1 – P4) used in the four-primer PCR analysis of transformant lines are indicated together with the expected product sizes. (**B**) PCR analysis of six transformant lines for each locus indicate that all are homoplasmic for the R1 plastome following three rounds of selection on Spc. The

WT band is the product of P1+P2, transformants bands are the product of P3+P2. (**C,D**) PCR analysis following two further rounds of restreaking on TAP or TAP+5-FC media demonstrates the stability of the R1 genotype in the absence of selection (P3+P2 product retained), and rapid loss of the *codA-aadA* cassette under 5-FC selection (new band corresponding to P4+P2 product). (**E**) Luminescence analysis of mid-log cultures of Luc1.R1, Luc1.R2, Luc2.R1 and Luc2.R2 lines. Luminescence units were normalised to optical cell density (OD_{750}) with values based on 3n replicates. (**F**) Spot tests of mid-log cultures and 1:10 and 1:100 dilutions of WT and transformant lines C-A, Luc1.R2 and Luc1.R2 on TAP plates containing: no antibiotic, Spc or 5-FC.

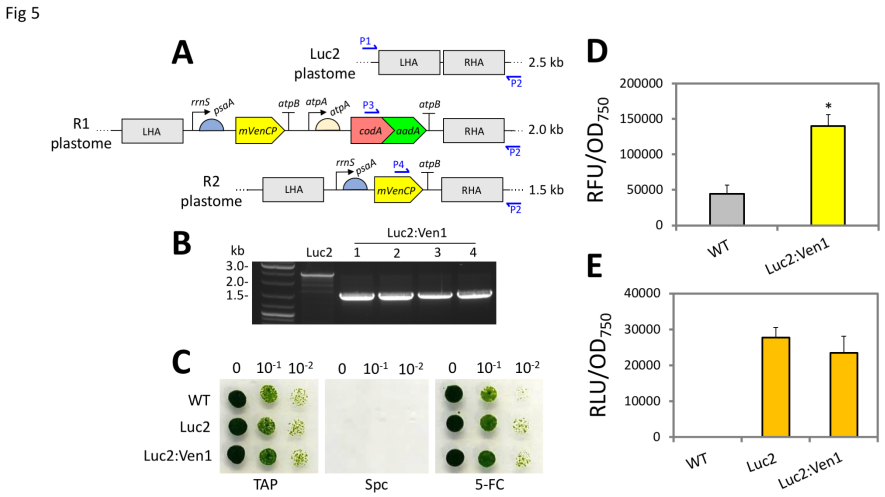

Figure 5: (**A**) Illustration of the plastome locus downstream of *psbA* prior to insertion of the mVenCP and*codA-aadA* cassettes (strain Luc2), as the intermediate recombinant locus (R1), and following loop-out of the *codA-aadA*marker (R2). The locations of the primers (P1 – P4) used in a four-primer PCR analysis of transformant lines are indicated together with the expected product sizes. (**B**) PCR conformation of homoplasmy at the R2 stage for four independent Luc2:Ven1 transformant lines. The Luc2 control showed the expected WT bands at 2.5 kb, whereas all four transformants showed the expected R2 PCR product at 1.5 kb. (**C**) Spot tests of mid-log cultures, and 1:10 and 1:100 dilutions, of WT, Luc2 and Luc2:Ven1 on TAP plates containing: no antibiotic, Spc or 5-FC. (**D**) Fluorescence analysis of Luc2:Ven1 compare to WT. Fluorescence measurements were taken on OD_{750} normalized mid-log cultures as three replicates (3n). (**E**) Microplate-based relative luminescence analysis of WT, Luc2 and Luc2:Ven1. Luminescence measurements were taken on OD_{750} normalized mid-log cultures as three replicates (3n).

9 Supplementary Information

File.S1

Description of modifications made to Start-Stop system and assembly strategy for level 2 constructs.

File.S2

Genbank format vectors maps for all constructs used in study.

Table.S1

Details of primers used for diagnostic PCR and establishment of homoplasmy.

Fig S1

SBOL representation of the plasmid constructs used to test the CpPosNeg strategy.