ACS Synthetic Biology

pubs.acs.org/synthbio Research Article

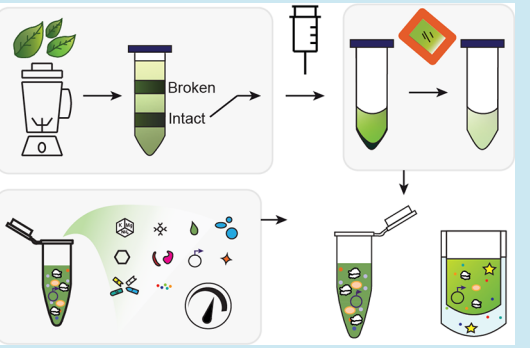
Establishing a High-Yield Chloroplast Cell-Free System for Prototyping Genetic Parts

Lauren Clark, Christopher A. Voigt, and Michael C. Jewett*

Cite This: ACS Synth. Biol. 2024, 13, 2402−2411 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Plastid engineering offers the potential to carry multigene traits in plants; however, it requires reliable genetic parts to balance expression. The difficulty of chloroplast transformation and slow plant growth makes it challenging to build plants just to characterize genetic parts. To address these limitations, we developed a high-yield cell-free system from *Nicotiana tabacum* chloroplast extracts for prototyping genetic parts. Our cell-free system uses combined transcription and translation driven by T7 RNA polymerase and works with plasmid or linear template DNA. To develop our system, we optimized lysis, extract preparation procedures (e.g., runoff reaction, centrifugation, and dialysis), and the physiochemical reaction conditions. Our cell-free system can synthesize 34 ± 1 μg/mL luciferase in batch reactions and 60 ± 4 μg/mL in semicontinuous reactions. We apply our batch reaction system to test a library of 103 ribosome binding site (RBS) variants and rank them based on cell-free gene expression. We observe a 1300-fold dynamic range of luciferase expression when normalized by maximum mRNA expression, as assessed by the malachite green aptamer. We also find that the observed normalized gene expression in chloroplast extracts and the predictions made by the RBS Calculator are correlated. We anticipate that chloroplast cell-free systems will increase the speed and reliability of building genetic systems in plant chloroplasts for diverse applications.



KEYWORDS: *chloroplast cell-free system, plant chloroplasts, Nicotiana tabacum, protein production, extract preparation*

INTRODUCTION

Engineered plant chloroplasts offer opportunities to synthesize pharmaceuticals, produce nutrients, and carry biosensors.[1] Plant genetic engineering is much more difficult and lengthier than bacterial genetic engineering (e.g., chloroplast transformation can take 6−12 months to achieve homoplasty and then seeds).[2] In addition, many species are not amenable to genetic manipulation and have limited sets of genetic tools.[3,4] This backdrop has resulted in a paradigm where useful genetic designs of plant origin are often ported into more tractable organisms such as *Escherichia coli* or *Chlamydomonas* for evaluation before going through the trouble of moving the pathway to a plant. However, nonplant systems differ in their molecular composition and regulatory signals, which can lead to inaccuracies in the data generated.

Cell-free gene expression (CFE) systems[5,6] have proven useful for accelerating biological design in the context of their native host organism's biological machinery.[7−15] For example, the development of CFE systems derived from a diverse set of organisms such as *Pseudomonas*,[16] *Streptomyces*,[17−21] *Vibrio natriegens*,[22,23] *Saccharomyces*,[24] *Clostridium*,[25] *Pichia pastoris*,[26] and Chinese Hamster Ovary[27,28] cells has opened new opportunities in rapid prototyping of synthetic biological systems, including genetic parts, such as promoters and RBSs.[15,16,29]

Here, we set out to advance CFE systems for chloroplasts. We focused on chloroplasts for several reasons. First, they are not subject to generational silencing, which can be a problem when carrying a genetic system in the plant nucleus. Second, chloroplasts are known for producing high titers of recombinant protein.[4,30−34] Third, chloroplast expression offers an inherent biocontainment[35,36] because chloroplasts are inherited maternally.[37] Finally, plastids have their own genome and bacteria-like ribosomes, which make them well-suited for cell-free expression.

As a model, we developed an optimized CFE platform from purified *Nicotiana tabacum* chloroplasts. This builds off decades of work in chloroplast molecular biology that have been a primary means of elucidating chloroplast genetics.[38−40] Previous works, for example, have shown the ability to purify chloroplasts from spinach and tobacco[41−45] and demonstrated the preparation of translation-only capable chloroplast extracts,[46,47] as well as *in vitro* transcription and translation.[41,42]

Received: February 17, 2024
Revised: May 22, 2024
Accepted: June 7, 2024
Published: July 18, 2024

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

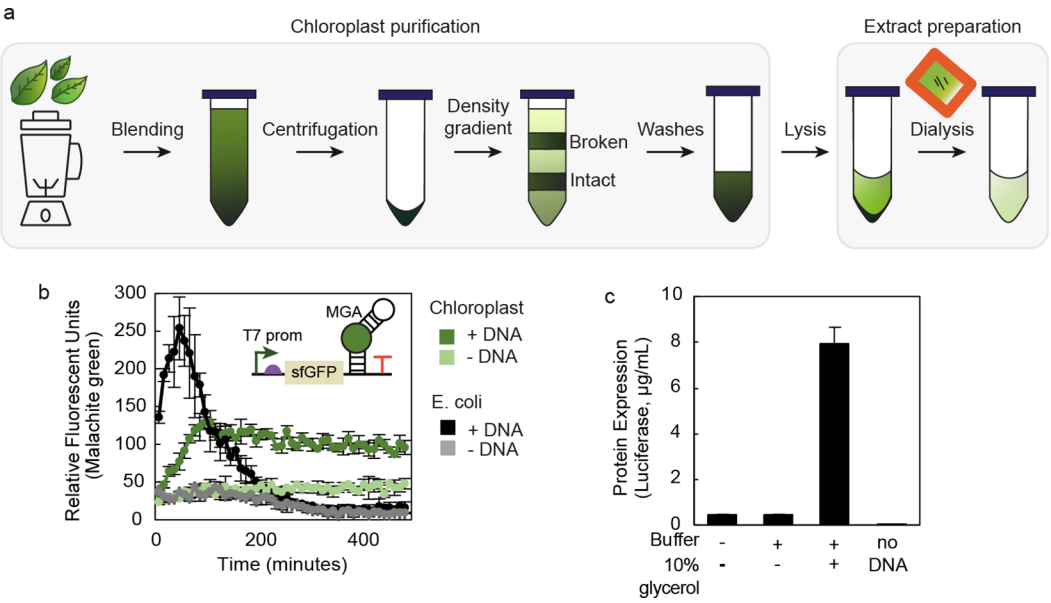


Figure 1. Establishing a cell-free transcription and translation system from *Nicotiana tabacum* chloroplasts. (a) Schematic of chloroplast purification and extract preparation. (b) Cell-free transcription of the malachite green RNA aptamer (MGA) from a T7 RNAP promoter in a CFE reaction containing the malachite green dye using either chloroplast or *E. coli* lysates and with or without plasmid DNA. (c) Combined cell-free transcription and translation reactions from chloroplast extracts are active. A no-DNA-negative control CFE reaction is shown (−DNA). Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

These *in vitro* systems proved useful for the analysis of promoter binding and transcription, leading to a greater understanding of chloroplast promoter architecture and polymerase-DNA binding.[38,39,48,49] Additionally, chloroplast extracts[50] and extract-based expression systems have been used to study chloroplast ribosome binding and understand the biology of translation initiation in the chloroplast (Table S1).[46,51−53] However, these previous cell-free systems have not been optimized for high-level or quantitative expression of reporter proteins to parametrize genetic parts.

We established a protocol for producing highly active chloroplast extracts, optimized the chemical reaction environment to increase CFE yields of reporter proteins, and applied this method to screen a library of RBSs designed for chloroplasts using the RBS Calculator.[54−59] We also discovered that chloroplast extracts maintain mRNA over long periods of time, which could be a useful feature for applications in testing circuits and sensors.

RESULTS

Activating High-Yield Protein Production in Cell-Free Chloroplast Extracts. The goal of this work was to develop a CFE system from *N. tabacum* chloroplasts capable of manufacturing reporter proteins for genetic part prototyping. We grew *N. tabacum* plants in Conviron growth chambers with a 16-h/8-h light−dark cycle at 28 °C.[47] Tobacco leaves were harvested at room temperature from plants at 6 weeks postgermination. Chloroplasts were purified from the three youngest, fully expanded leaves using a blender, cloth straining, density centrifugation, and washes at 4 °C. After purification, chloroplasts were flash frozen and stored at −80 °C. Then, chloroplasts were thawed on ice and resuspended in lysis buffer, lysed via syringe, and extracts were processed based on previous protocols for translation-only extracts (Figure 1A).[46,47] Processing steps postlysis included a runoff reaction consisting of incubation for 15 min at 28 °C, centrifugation to remove membrane material, dialysis, and a final centrifugation. These extracts had 32 ± 5 mg/mL of total chloroplast protein and were subsequently tested for activity in cell-free transcription and translation.

We first carried out cell-free transcription in a reaction mixture containing a DNA template, exogenously added T7 RNA polymerase (RNAP), energy substrates, nucleotides, and salts necessary for gene expression.[47,60] We assessed transcription levels with a reporter template that harbored the malachite green aptamer (MGA) sequence (Figure 1B). Once transcribed, this RNA aptamer binds to malachite green and activates the dye's fluorescence.[61,62] mRNA concentrations remain stable for over 20 h in chloroplast extracts. This contrasts with *E. coli* extracts, where RNA increases rapidly, peaks, and degrades (Figure 1B), as has been observed before.[15,63] The mRNA stability in chloroplast extracts could be a result of the presence of pentatricopeptide proteins, which contain RNA binding motifs. These proteins have been identified in the chloroplast and are thought to protect mRNA templates *in vivo*.[64−66] It is also worth noting that mRNA half-lives are on the order of hours in chloroplasts, while they are on the order of minutes in bacteria.[67,68]

We then assessed the combined transcription and translation of a luciferase reporter protein. Luciferase was selected because bioluminescence assays are highly sensitive with virtually no background and could be useful for genetic part prototyping. Unfortunately, our early protein expression levels were low (~0.4 μg/mL) (Figure 1C). In making extracts for those reactions, we directly froze the chloroplast pellets after purification but before lysis (Figure 1C, buffer control). We worried that chloroplasts might break open after thawing but prior to extract preparation (Figure 1A), reducing the translation machinery available for protein production in prepared extracts. Therefore, we next resuspended chloroplasts in lysis buffer or lysis buffer with 10% glycerol prior to freezing in liquid nitrogen and extract preparation (Figure 1C).

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

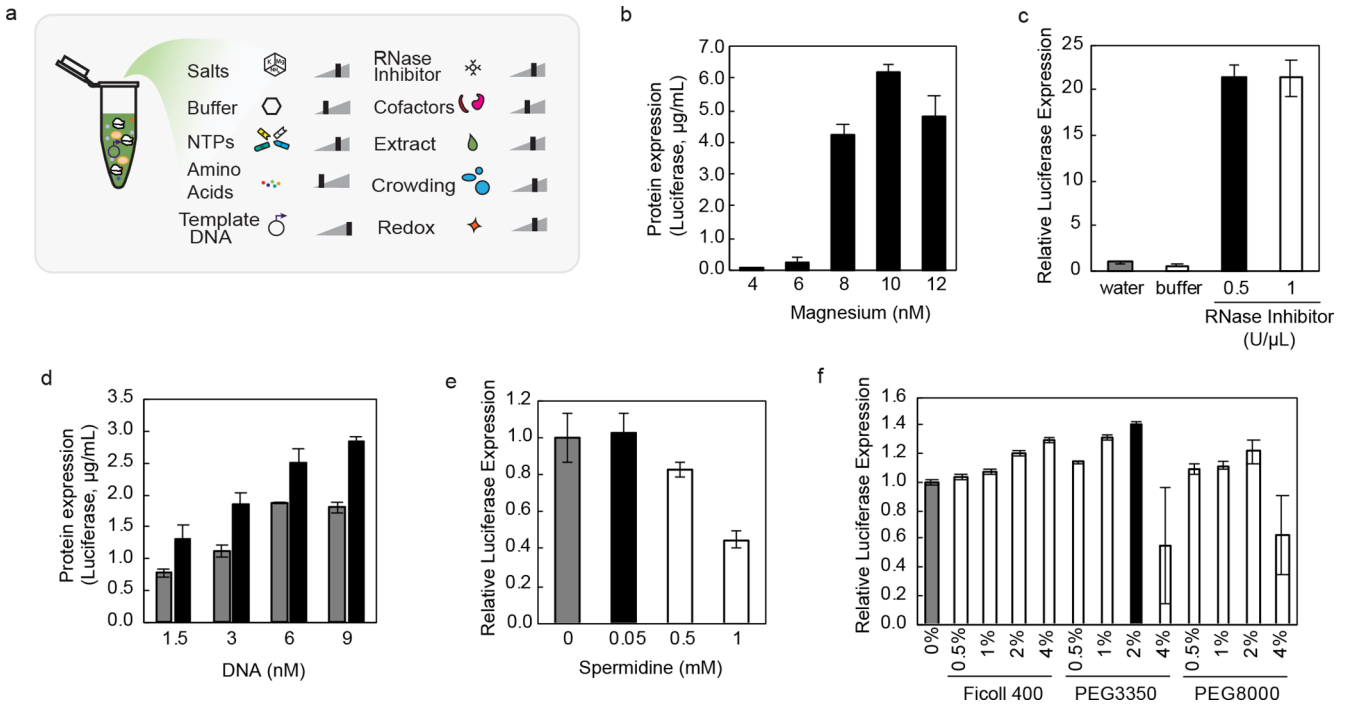


Figure 2. Physiochemical optimization. (a) The cell-free environment was optimized by testing a range in concentrations of several reaction components. Triangles indicate experimental tuning of the cell-free reaction environment for maximum productivity (e.g., high to low RNase inhibitor concentration). These components include (b) magnesium acetate, (c) RNase inhibitor, (d) DNA in the form of plasmids (gray bars) or linear expression templates (LETs, black bars), (e) spermidine, and (f) crowding agents. Notably, magnesium is optimized as a quality control step for each independent extract. In panels c, e, and f, gray bars indicate the condition prior to optimization and black bars indicate the condition used in all future experiments. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments (n = 3).

Glycerol can act as a cryoprotectant to protect chloroplasts from freezing damage and premature rupture. We found that resuspending chloroplasts in a 10% glycerol lysis buffer yielded extracts with 30% higher total protein concentrations (42 ± 2 mg/mL) and improved CFE productivity 18-fold. Protein expression yields could be further improved by adjusting codon usage of our pJL1 luciferase reporter,[69] which was originally created for bacterial expression, to be optimized for chloroplast expression (Figure S1). We used this optimized coding sequence and chloroplasts prepared in glycerol for all further cell-free reactions.

Optimizing the Cell-Free Reaction Environment. We next sought to increase protein synthesis yields by systematically optimizing the physiochemical reaction environment (Figure 2A). This was important because the physiochemical conditions of cell-free reactions are known to play a key role in the operation of *in vitro* biological processes.[70−72]

Our initial physiochemical optimizations focused on the salt concentrations, buffer, extract amount, and reduction potential. We started with salts and specifically magnesium, which has been previously shown to be a critical component of CFE reactions.[70] Our data suggested an optimum magnesium concentration of 10 mM magnesium acetate (Figure 2B). However, we later found that reoptimization of magnesium acetate was needed for each batch of extract, which is typical in the field.[17,27,70,73] After magnesium, we optimized potassium, which is used as the major cation of the system, as well as ammonium, which is used to mimic the natural environment. We found that 60 mM potassium acetate and 30 mM ammonium acetate led to the highest CFE yields (Figure S2). Next, we explored a range of buffer concentrations (HEPES 15−120 mM). Our results indicated that the 15 mM HEPES at pH 7.3 led to the highest translational activity. Finally, we assessed the impact of the percent extract volume and the reducing agent DTT, settling on a 50% volume fraction and 5 mM DTT (Figure S3).

We next explored components involved in supporting high-level transcription and translation. We focused on protein coding templates in the form of DNA and RNA. First, we tested adding RNase inhibitors to our CFE reactions. We observed a dramatic improvement in cell-free activity with 0.5 U/μL RNase inhibitor (Figure 2C). Second, by titrating different concentrations of plasmid DNA, we observed that we could increase yields (Figure 2D). Given robust expression with plasmid DNA, we also assayed linear DNA expression templates (LETs) (i.e., PCR products) at the same molarity that we used for plasmid DNA. Relative to plasmid DNA, PCR products have the benefit of avoiding cloning steps and thus can expedite high-throughput workflows for validating genetic part performance *in vitro*. We found that we could increase luciferase yields by over 50% as compared to when plasmid DNA was used as a template (Figure 2D). Third, we assessed the impact of spermidine on the system, as this polycation has been shown to stabilize DNA, RNA, and tRNA and aid in T7 RNAP function.[74−76] While spermidine did not statistically increase yields (Figure 2E), we elected to add 0.05 mM in subsequent reactions to better mimic cellular physiochemical conditions.[70] Finally, we assayed a panel of crowding agents given that these impart molecular crowding effects that can enhance transcription and translation activity.[61] We tested a range of concentrations of Ficoll 400, PEG 3350, and PEG 8000 from 0.5% to 4% (v/v) and saw the biggest improvement

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

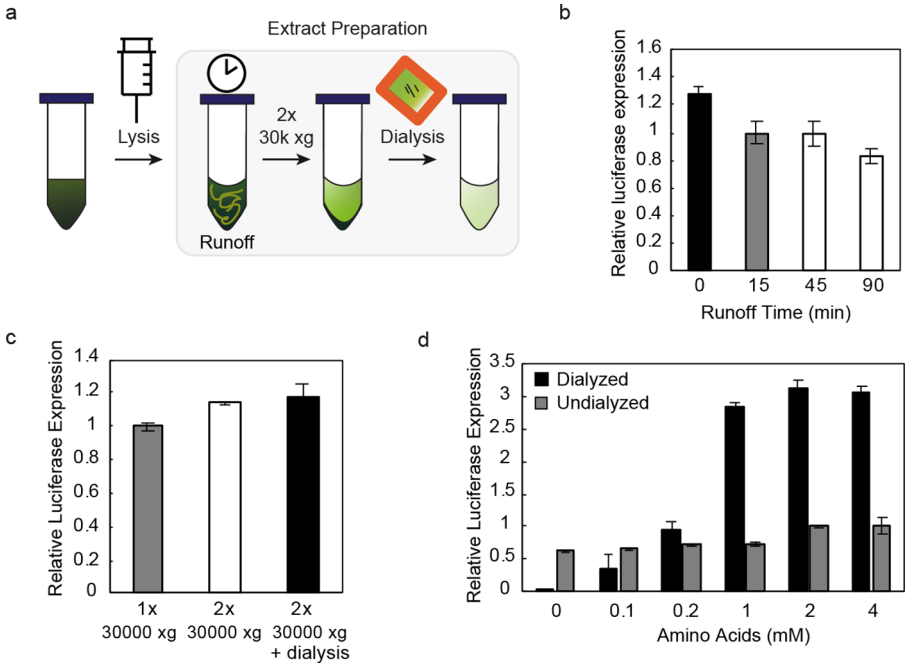


Figure 3. Optimization of extract preparation procedures and amino acid concentrations. (a) Cartoon schematic of the finalized extract preparation procedure. Our initial extract preparation was modified to remove the runoff incubation, including a second 30 000 × *g* spin, and dialysis. To improve cell-free protein synthesis yields, we optimized extract preparation procedures including (b) runoff reaction, (c) centrifugation and dialysis, and (d) amino acids. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments (n = 3).

from PEG 3350 at 2% v/v (Figure 2F). Table S2 shows the physiochemical conditions of the final optimized chloroplast CFE system as compared to other relevant cell-free systems.

Optimizing Cell-Free Extract Production. With new physiochemical conditions at hand, we set out to optimize extract preparation procedures to further increase cell-free protein biosynthesis yields. Our initial procedure consisted of chloroplast lysis, a runoff incubation, and centrifugation at 30 000 × *g* (Figure 1A). However, these conditions were not optimized. Here, we examined these extract preparation conditions, including the addition of a dialysis step, starting with the runoff reaction (Figure 3A).

The runoff reaction, or incubation of the lysate with substrates necessary for translation, was selected for optimization first because it is hypothesized to release actively translating ribosomes from mRNA, freeing them to translate recombinant transcripts. In the case of chloroplasts, these ribosomes may be anchored to the thylakoid membrane, translating the abundant membrane-bound RuBisCo proteins. Despite hypothesizing that the runoff reaction might increase yields, we found that omitting any incubation postlysis was the most beneficial condition (Figure 3B).

After removing the runoff reaction step, we found that the chloroplast membrane material pelleted loosely in the glycerol buffer. Thus, we assessed the incorporation of a second centrifugation step at 30 000 × *g* to eliminate membrane material more easily during extract preparation. We observed a slight increase in protein expression yields (Figure 3C), as well as more robust extract preparation with this step and so it was subsequently always used.

We next explored the impact of dialysis, which is commonly used in CFE protocols to remove metabolic byproducts and provide a suitable storage buffer.[61] Dialysis was carried out at 4 °C in 10% glycerol lysis buffer (Figure 3C). Following dialysis, we conducted a final study to optimize amino acids for the chloroplast CFE reaction. We observed that amino acid concentrations higher than 1 mM were important for achieving high yields in dialyzed extracts (Figure 3D), indicating that dialysis seems to improve the translational capacity of this cell-free system.

By optimizing chloroplast lysis conditions, the physiochemical reaction environment, and extract processing methods, we improved protein synthesis yields more than 100-fold relative to our first starting conditions (Figure 4A). Figure 4B shows active luciferase yield throughout the duration of the cell-free batch reaction. The final yield of luciferase after a 24-h incubation in a batch CFE reaction was 34 ± 1 μg/mL.

We then applied semicontinuous cell-free protein synthesis reaction[77] using a dialysis device (3.5 K MWCO) to increase luciferase production yield. Batch cell-free reactions include all

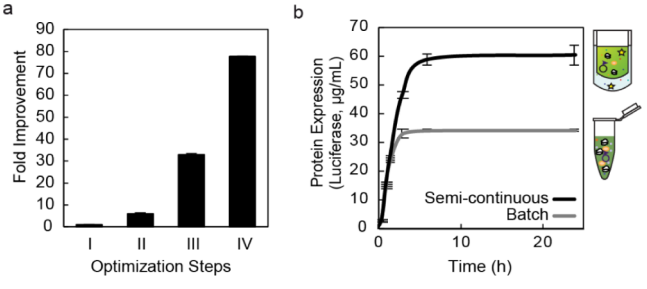


Figure 4. Analysis of improvements and maximum cell-free expression yields. (a) Cumulative improvements of cell-free expression over the process and physiochemical optimizations. I. Initial system. II. Freezing and lysis in the presence of 10% glycerol lysis buffer. III. Physiochemical optimization as referenced in Table S2. IV. Removal of runoff reaction and addition of dialysis. (b) Reaction dynamics of batch and semicontinuous reaction modes. Values show means with error bars representing standard deviations (s.d.) of at least three independent experiments.

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

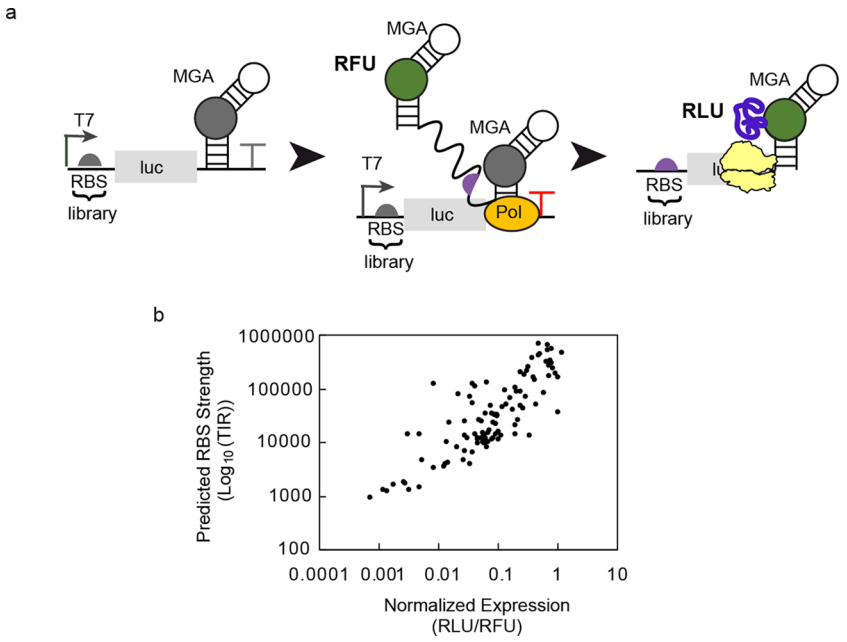


Figure 5. The chloroplast cell-free system can be used to rank genetic parts. (a) Cartoon schematic of the RBS library assay. (b) We compared predicted RBS strength from the RBS calculator versus normalized gene expression, or proteins per transcripts (maximum luminescence/maximum fluorescence), in the chloroplast cell-free system. Values show means of at least three independent experiments (n = 3) with normalized error reported in Figure S5.

reaction components in a closed vessel. On the other hand, semicontinuous reactions are traditional batch reactions set up in a dialysis vessel that is placed in a buffer containing the cell-free reaction components with no extract or DNA. This outer buffer is present in a greater volume than the inner reaction, allowing any deleterious byproducts to diffuse away into the buffer and any reagents and cofactors that are consumed to be replenished in the reaction due to the presence of a semipermeable membrane between the reaction and the buffer. With a semicontinuous setup, luciferase production was increased to 60 ± 4 μg/mL, which was nearly double the yield of batch reaction conditions (Figure 4B). The time-course reactions show that this chloroplast system can produce a stable, active protein with little apparent degradation in the case of the enzyme luciferase.

Analysis of an RBS Library with Chloroplast CFE. We measured the strengths of RBSs. We designed 103 RBSs replacing the original RBS sequence between the T7 promoter sequence and the start of the luciferase gene (Figure 5A). The MGA followed the luciferase gene to allow estimation of mRNA concentration. The RBS designs were predicted by the RBS calculator[54−59] with a wide spectrum of expression levels (Figure S4) using the predicted sequence of AAGGAGVBHD-HYBD for the chloroplast RBS and spacer region. This sequence was sufficiently variable to generate thousands of sequences, while still containing the canonical core sequence of the tobacco chloroplast 16S rRNA.[78] For clarity, the canonical chloroplast RBS is GGAGG and the canonical *E. coli* RBS is AGGAGG. *E. coli* was used as an organism to predict the expression level by the RBS Calculator. The resulting library covered a 3-order-of-magnitude range in predicted translation initiation rates (TIRs) from maximal (RBS sequence 1) to minimal (RBS sequence 103) (Table S3).

We carried out cell-free gene expression reactions and assessed both transcription (MGA) and translation (luciferase) activities (Figure 5). We observed a 1300-fold range of normalized luciferase expression (proteins/transcripts; relative luminescence units (RLU)/relative fluorescence units (RFU)). We also found that the predicted RBS TIR and luciferase yield normalized by mRNA transcript levels trended together (Figure 5B). Separated transcription and translation data are found in Figure S5.

DISCUSSION

We developed a cell-free gene expression system from *Nicotiana tabacum* chloroplasts capable of producing the easy-to-use reporter protein luciferase. Transcription was obtained by the exogenous T7 RNAP. Obtaining sufficient reporter levels was achieved by optimizing plant growth and lysis, the physiochemical reaction conditions, and extract preparation procedures (e.g., runoff reaction, centrifugation, and dialysis). A key insight was the use of glycerol to stabilize the chloroplasts prior to lysis. Protein biosynthesis yields of luciferase for the best-performing batch CFE system were 34 ± 1 μg/mL.

Our chloroplast-based system is unique from the previously published tobacco BY-2 cell lysate system,[79,80] which is a highly productive expression system capable of combined transcription, translation, and protein modification. Specifically, the BY-2 lysate system is a eukaryotic chromosomal system and thus is different.

The CFE system was applied to screen a library of 103 RBSs in less than 1 day that were computationally designed using the RBS calculator. We observed a correlation between the RBS calculator predictions of TIR and normalized cell-free gene expression when adjusted for transcription. The library we produced showed a 3-order-of-magnitude dynamic range of proteins per transcript between the lowest expressing and the highest expressing sequences in the chloroplast CFE system, demonstrating a system well suited to ranking DNA templates for plant engineering purposes.

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

While predicted and actual gene expression were correlated, some differences also existed. These differences could be due to several reasons, such as whether the plant is exposed to light or dark during growth. For example, the *psbA* transcript, a part of the photosystem II complex, is the most actively translated mRNA in light-grown conditions *in vivo*, yet it is not so in the dark or in nonphotosynthetic plastids.[46] Our workflow was developed using light-harvested chloroplasts; modifications to the system including possible reoptimization are needed for the study of nighttime translational programs. Additionally, regulation in plastids occurs at the transcript level by RNases, and it is not currently known how much of this machinery is present or active in these extracts.

We did not observe transcriptional activity in the chloroplast CFE system when evaluating promoters transcribed by endogenous RNAPs (data not shown). Chloroplast extract preparation procedures could be further modified to explore these phenomena and develop strategies to activate endogenous transcription.

Our work provides a platform for prototyping plant-based genetic parts in a chloroplast CFE system before evaluating smaller design sets in cells, as has been done in a variety of cell-free systems.[14,15,81−83] We anticipate the chloroplast CFE system will accelerate the characterization of reliable genetic parts for plant synthetic biology.

METHODS

Growth Conditions. *Nicotiana tabacum* var. Bright Yellow (PI 552 597) were acquired from GRIN-GLOBAL and grown on Metro-Mix 360 from SunGro via Fosters, Inc. (Waterloo, IA, USA) at 28 °C under 16-h white light/8-h dark conditions in a Conviron A1000 growth chamber for 6 weeks postgermination.

Purification of Chloroplasts. Chloroplast purification was adapted from previous work.[47] The top 3 healthy leaves below the apical leaf were removed from 6-week-old plants exposed to 6−7 h light. 300 g leaves were collected and blended in 100 g batches. Leaves were removed to 4 °C for the remainder of the protocol. Leaves were ripped into 4−6 pieces each and loaded into a Waring blender. 300 mL buffer MCB1 (50 mM HEPES/KOH, pH 8.0, 0.3 M mannitol, 2 mM EDTA, 5 mM β-mercaptoethanol) with 0.1% w/v BSA and 0.6% w/v polyvinylpyrrolidone (average molecular weight 40 000) was poured over the leaves, and the blender was run on high for two 5-s intervals and then a 2-s interval, checking the blending at each pause to ensure all leaves are destroyed. The brei was then combined and strained with two sheets each of cheesecloth and Miracloth (EMD Millipore, Burlington, MA, USA) and centrifuged at 1000× *g*. After centrifugation, the pellet was resuspended in 18 mL of MCB1 with 0.1% BSA, and 2−4.5 mL of material was layered onto stepwise Percoll (GE Healthcare, Chicago, IL, USA) MCB1 gradients with 0.1% BSA. Per 300 g leaves, 10 gradients were prepared with a Hamilton syringe with 7 mL of 20% Percoll, 12 mL of 50% Percoll, and 11 mL of 80% Percoll. Loaded Percoll gradients were centrifuged in a fixed-angle rotor for 10 min with minimum acceleration and deceleration, and the green band between the 50% and 80% phases was collected as intact chloroplasts. Chloroplasts were washed three times in MCB2 (50 mM HEPES/KOH, pH 8.0, 0.32 M mannitol, 2 mM EDTA, 5 mM β-mercaptoethanol). The first wash was in 3:1 volumes of buffer to chloroplast material, the second wash was in 60 mL, and the final wash was in 8 mL. To collect chloroplasts between washes, chloroplasts were centrifuged at 1000× *g* for 4 min, and after the final wash, they were centrifuged at 5000× *g* for 4 min. Between washes, resuspension was done by gentle swirling motion by hand to avoid lysing the chloroplasts. After the final wash, chloroplasts were resuspended by gentle pipetting in 1 mL/g lysis buffer (30 mM HEPES/KOH, pH 7.7, 60 mM potassium acetate, 7 mM magnesium acetate, 60 mM ammonium acetate, 10% v/v glycerol, 5 mM DTT, 20 μM each of 20 amino acids, 0.1 mM GTP, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), where the amino acids and GTP were added after thawing, flash-frozen, and then stored at −80 °C.

S30 Preparation of Extract. All procedures were carried out at 4 °C or on ice. Unless otherwise noted, frozen chloroplasts in lysis buffer were thawed on ice for 20 min and then mixed by pipetting. Chloroplasts were lysed by passing through a 25G syringe 12 times and centrifuged at 30 000× *g* at 4 °C for 30 min. The supernatant was removed and centrifuged a second time at 30 000× *g* at 4 °C for 30 min. The supernatant from the second spin was loaded into a dialysis cassette (Slide-A-Lyzer 10K MWCO, Pierce Biotechnologies, Waltham, MA, USA), dialyzed twice for 2 h each against 600 mL of buffer with 30 mM HEPES/KOH, pH7.7, 60 mM potassium acetate, 7 mM magnesium acetate, 60 mM ammonium acetate, 10% v/v glycerol, 5 mM DTT, and 0.5 mM PMSF, and centrifuged a final time at 4 °C at 30 000× *g* for 20 min. The supernatant was removed and aliquoted, then flash-frozen, and stored at −80 °C.

Cell-Free Protein Synthesis Reaction. Reactions were run at 25 °C in 10 μL of total volume in 1.5 mL Eppendorf tubes or in 384-well plates for Malachite green assays. Reactions were assembled on ice from stock solutions within the ranges described in Table 2, with most reactions run (unless otherwise noted) with 15 mM HEPES pH 7.3, 60 mM potassium acetate, 4−10 mM magnesium acetate, 30 mM ammonium acetate, 2 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM UTP, 2 mM each of 20 amino acids, 8 mM creatine phosphate, 5 mM DTT, 0.05 mM spermidine, 2% w/v PEG 3350, 0.5 U/uL RNase inhibitor (Clontech, Mountain View, CA, USA), 0.1 mg/mL T7 polymerase (made in house following the protocol from Swartz et al., 2004),[84] 0.33 mg/mL creatine phosphokinase (from rabbit muscle, Sigma-Aldrich), 9 nM plasmid or linear DNA, and 50% v/v S30 extract. Reactions were run overnight at room temperature. Plasmid DNA was prepared using the ZymoPure II Plasmid Midiprep Kit followed by ethanol precipitation, and linear expression templates were prepared by PCR and subsequent cleanup using the Zymo DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA).

The amount of active firefly luciferase produced was quantified by activity test. Four microliters of CFPS sample were added to 30 μL of ONE-Glo Luciferase Assay System (Promega, Madison WI, USA) in a white 96-well plate (Costar 3693, Corning, Corning, NY, USA). Luminescence was measured at 26 °C every 2 min for 20 min using a BioTek (Winooski, VT, USA) Synergy 2 plate reader. For each reaction, the maximum relative light units (RLU) were used to compare to a linear standard curve of recombinant luciferase taken under the same conditions in cell-free buffer added to the ONE-Glo mixture.

mRNA Detection with Malachite Green Assay. Reactions were assembled in triplicate in 10 μL volumes on ice as described above with 0.02 mM malachite green dye, 30

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

mM HEPES pH 7.7, 60 mM potassium acetate, 10 mM magnesium acetate, 60 mM ammonium acetate, 2 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM UTP, 0.1 mM each of 20 amino acids, 8 mM creatine phosphate, 5 mM DTT, 0.1 mg/mL T7 polymerase (made in house following the protocol from Swartz et al., 2004),[84] 0.33 mg/mL creatine phosphokinase (from rabbit muscle, Sigma-Aldrich C3755−3.5KU), 3 nM plasmid DNA, or water as a control for background signal and 50% v/v S30 extract. Bacterial reactions were assembled as described in Silverman et al. (2019)[61] with added T7 polymerase.

Kinetic cell-free reactions were assembled on ice in triplicate and pipetted into a 384-well plate (Grenier BioOne 781 096) avoiding bubbles. Plates were sealed (Bio-Rad MSB1001) and both sfGFP fluorescence (emission/excitation: 485/528, gain 50) and malachite green fluorescence (emission/excitation: 615/650, gain 100) were measured every 10 min overnight for 8 h at 25 °C on a BioTek Synergy H1M plate reader.

Codon Optimization and Plasmid Construction. Codon optimization was conducted on our in-house firefly luciferase sequence by hand based on previous work.[85] Residues N, D, A, Y, and F were optimized based on reported relative translation efficiencies, and all other residues were optimized based on tobacco chloroplast codon usage. Some residues without reported translation rates were left unoptimized to allow for synthesis by Integrated DNA Technologies. Inserts were ordered from Integrated DNA Technologies (Coralville, IA) and cloned into the pJL1 backbone by the method described in Gibson et al.[86] Inserts included an overhang of anywhere from 27 to 73 nucleotides with the NdeI and *Sal*I cut sites on pJL1 to facilitate Gibson assembly without the need for primers.

RBS Library Design and Construction. RBSs were designed with the RBS Library Calculator in predict mode with the following sequence (aataattttgtttaactttaagaaggagVBHDHYBD). Of the 4374 variants designed, 103 were selected that sampled the range of the transcription initiation rate of the designs. These were ordered from Twist Bioscience as variations on the pJL1-lucME plasmid with the malachite green aptamer on the 3′ end. Library members were amplified by PCR, purified with a Zymo ZR-96 Clean and Concentrator kit, and quantified with the Promega QuantiFluor kit. All DNAs were then diluted to an end dilution of 5.63 nM and tested as linear expression templates in cell-free reactions in 384-well plates as described above, except that reactions were incubated for 20 h and optimized cell-free conditions were used for the chloroplast reactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.4c00111.

Supplementary FiguresS1−S5 describe the reporter luciferase gene optimization, chloroplast-based CFE-reactions, predicted RBS strengths, and cell-free transcription or translation.Supplementary Tables S1−S3 contain example extract-based systems producedfrom plant chloroplasts, physiochemical optimization ranges of typicalcell-free systems, and RBS used in this study (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Michael C. Jewett − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Chemistry of Life Processes Institute, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States; Robert H. Lurie Comprehensive Cancer Center, Chicago, Illinois 60611, United States; Simpson Querrey Institute, Northwestern University, Chicago, Illinois 60611, United States; Department of Bioengineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0003-2948-6211; Phone: 1 847 467 5007; Email: m-jewett@northwestern.edu; Fax: 1 847 491 3728

Authors

Lauren Clark − *Department of Chemical and Biological Engineering, Northwestern University, Evanston, Illinois 60208, United States; Chemistry of Life Processes Institute, Evanston, Illinois 60208, United States; Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, United States*

Christopher A. Voigt − *Synthetic Biology Center, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States;* orcid.org/0000-0003-0844-4776

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.4c00111

Author Contributions

C.A.V. and M.C.J. conceptualized and supervised the study. L.G.C. and M.C.J. designed the study. L.G.C. performed and analyzed all experiments. L.G.C. and M.C.J. wrote the manuscript. All authors edited and reviewed the manuscript.

Notes

The authors declare the following competing financial interest(s): L.C., C.A.V., and M.C.J. are co-inventors on provisional patent applications that incorporate discoveries described in this manuscript. M.C.J. is a cofounder of SwiftScale Biologics, Stemloop, Inc., Pearl Bio, and Synolo Therapeutics. M.C.J.s interests are reviewed and managed by Northwestern University and Stanford University in accordance with their conflict-of-interest policies. All other authors declare no competing interests.

■ ACKNOWLEDGMENTS

L.C. thanks Yasushi Yukawa for guidance on chloroplast purification and lysis, Eszter Majer for fruitful discussions, and Holly Ekas for guidance on high-throughput DNA quantification and purification for cell-free applications. M.C.J. and C.A.V. were funded by the DARPA 1KM program [HR0011-15-C-0084]. We thank Ashty Karim for his help in editing the manuscript.

■ REFERENCES

(1) Liu, W.; Stewart, C. N. Plant synthetic biology. *Trends Plant Sci.* **2015**, *20*, 309−317.

(2) Ruf, S.; et al. High-efficiency generation of fertile transplastomic Arabidopsis plants. *Nat. Plants* **2019**, *5*, 282−289.

(3) Bock, R. Genetic engineering of the chloroplast: Novel tools and new applications. *Curr. Opin. Biotechnol.* **2014**, *26*, 7−13.

(4) Yu, Y.; Yu, P.-C.; Chang, W.-J.; Yu, K.; Lin, C.-S. Molecular Sciences Plastid Transformation: How Does it Work? Can it Be Applied to Crops? What Can it Offer? *Int. J. Mol. Sci.* **2020**, *21*, 4854.
(5) Kightlinger, W.; Duncker, K. E.; Ramesh, A.; Thames, A. H.; Natarajan, A.; Stark, J. C.; Yang, A.; Lin, L.; Mrksich, M.; DeLisa, M. P.; Jewett, M. C.; et al. A cell-free biosynthesis platform for modular construction of protein glycosylation pathways. *Nat. Commun.* **2019**, *10* (1), 5404.
(6) Jaroentomeechai, T.; Stark, J. C.; Natarajan, A.; Glasscock, C. J.; Yates, L. E.; Hsu, K. J.; Mrksich, M.; Jewett, M. C.; DeLisa, M. P.; et al. Single-pot glycoprotein biosynthesis using a cell-free transcription-translation system enriched with glycosylation machinery. *Nat. Commun.* **2018**, *9* (1), 2686.
(7) Hunt, A. C.; Vögeli, B.; Hassan, A. O.; Guerrero, L.; Kightlinger, W.; Yoesep, D. J.; Krüger, A.; DeWinter, M.; Diamond, M. S.; Karim, A. S.; Jewett, M. C.; et al. A rapid cell-free expression and screening platform for antibody discovery. *Nat. Commun.* **2023**, *14* (1), 3897.
(8) Eric Liew, F.; Nogle, R.; Abdalla, T.; Rasor, B.J.; Canter, C.; Jensen, R.O.; Wang, L.; Strutz, J.; Chirania, P.; De Tissera, S.; Mueller, A.P.; et al. Carbon-negative production of acetone and isopropanol by gas fermentation at industrial pilot scale. *Nat. Biotechnol.* **2022**, *40*, 335−344.
(9) Kightlinger, W.; et al. Design of glycosylation sites by rapid synthesis and analysis of glycosyltransferases article. *Nat. Chem. Biol.* **2018**, *14*, 627−635.
(10) Karim, A. S.; et al. In vitro prototyping and rapid optimization of biosynthetic enzymes for cell design. *Nat. Chem. Biol.* **2020**, *16*, 912−919.
(11) Bogart, J. W.; et al. Cell-Free Exploration of the Natural Product Chemical Space. *ChemBiochem* **2021**, *22*, 84−91.
(12) Dudley, Q. M.; Karim, A. S.; Nash, C. J.; Jewett, M. C. In vitro prototyping of limonene biosynthesis using cell-free protein synthesis. *Metab. Eng.* **2020**, *61*, 251−260.
(13) Kopniczky, M. B.; et al. Cell-Free Protein Synthesis as a Prototyping Platform for Mammalian Synthetic Biology. *ACS Synth. Biol.* **2020**, *9*, 144−156.
(14) Moore, S. J.; MacDonald, J. T.; Freemont, P. S. Cell-free synthetic biology for in vitro prototype engineering. *Biochem. Soc. Trans.* **2017**, *45*, 785−791.
(15) Moore, S. J.; MacDonald, J. T.; Wienecke, S.; Ishwarbhai, A.; Tsipa, A.; Aw, R.; Kylilis, N.; Bell, D. J.; McClymont, D. W.; Jensen, K.; Polizzi, K. M.; et al. Rapid acquisition and model-based analysis of cell-free transcription−translation reactions from nonmodel bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115* (19), 4340−4349.
(16) Wang, H.; Li, J.; Jewett, M. C. Development of a Pseudomonas putida cell-free protein synthesis platform for rapid screening of gene regulatory elements. *Synth. Biol.* **2018**, *3*, 1−7.
(17) Li, J.; Wang, H.; Kwon, Y.-C.; Jewett, M. C. Establishing a high yielding *streptomyces* -based cell-free protein synthesis system. *Biotechnol. Bioeng.* **2017**, *114*, 1343−1353.
(18) Li, J.; Wang, H.; Jewett, M. C. Expanding the palette of Streptomyces-based cell-free protein synthesis systems with enhanced yields. *Biochem. Eng. J.* **2018**, *130*, 29−33.
(19) Xu, H.; Liu, W. Q.; Li, J. Translation related factors improve the productivity of a streptomyces-based cell-free protein synthesis system. *ACS Synth. Biol.* **2020**, *9*, 1221−1224.
(20) Liu, W. Q.; Zhang, L.; Chen, M.; Li, J. Cell-free protein synthesis: Recent advances in bacterial extract sources and expanded applications. *Biochem. Eng. J.* **2019**, *141*, 182−189.
(21) Moore, S. J.; Lai, H. E.; Needham, H.; Polizzi, K. M.; Freemont, P. S. Streptomyces venezuelae TX-TL − a next generation cell-free synthetic biology tool. *Biotechnol. J.* **2017**, *12*, 1600678.
(22) Des Soye, B. J.; Davidson, S. R.; Weinstock, M. T.; Gibson, D. G.; Jewett, M. C. Establishing a High-Yielding Cell-Free Protein Synthesis Platform Derived from Vibrio natriegens. *ACS Synth. Biol.* **2018**, *7*, 2245−2255.
(23) Wiegand, D. J.; Lee, H. H.; Ostrov, N.; Church, G. M. Establishing a Cell-Free Vibrio natriegens Expression System. *ACS Synth. Biol.* **2018**, *7*, 2475−2479.
(24) Hodgman, C. E.; Jewett, M. C. Optimized Extract Preparation Methods and Reaction Conditions for Improved Yeast Cell-Free Protein Synthesis. *Biotechnol. Bioeng.* **2013**, *110*, 2643−2654.
(25) Krüger, A.; et al. Development of a clostridia-based cell-free system for prototyping genetic parts and metabolic pathways. *Metab. Eng.* **2020**, *62*, 95−105.
(26) Aw, R.; Spice, A. J.; Polizzi, K. M. Methods for Expression of Recombinant Proteins Using a Pichia pastoris Cell-Free System. *CP Protein Science* **2020**, *102*, 115.
(27) Martin, R. W.; et al. Development of a CHO-Based Cell-Free Platform for Synthesis of Active Monoclonal Antibodies. *ACS Synth. Biol.* **2017**, *6*, 1370−1379.
(28) Thoring, L.; Kubick, S. Versatile cell-free protein synthesis systems based on chinese hamster ovary cells. In *Recombinant Protein Expression in Mammalian Cells: Methods and Protocols*; Humana Press Inc.; 2018, Vol. *1850*, pp. 289308.
(29) E. Vezeau, G.; M. Salis, H. Tuning Cell-Free Composition Controls the Time Delay, Dynamics, and Productivity of TX-TL Expression. *ACS Synth. Biol.* **2021**, *10*, 2508−2519.
(30) Oey, M.; Lohse, M.; Kreikemeyer, B.; Bock, R. Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J.* **2009**, *57*, 436−445.
(31) Oey, M.; Lohse, M.; Scharff, L. B.; Kreikemeyer, B.; Bock, R. Plastid production of protein antibiotics against pneumonia via a new strategy for high-level expression of antimicrobial proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 6579−6584.
(32) Zhou, F.; et al. High-level expression of human immunodeficiency virus antigens from the tobacco and tomato plastid genomes. *Plant Biotechnol J.* **2008**, *6*, 897−913.
(33) Boyhan, D.; Daniell, H. Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant Biotechnol J.* **2011**, *9*, 585−598.
(34) Daniell, H.; Singh, N. D.; Mason, H.; Streatfield, S. J. Plant-made vaccine antigens and biopharmaceuticals. In *Trends in Plant Science*, 2009; Vol. *14*, pp. 669679. .
(35) Ruf, S.; Karcher, D.; Bock, R. Determining the transgene containment level provided by chloroplast transformation. *Proc. Natl. Acad. Sci.* **2007**, *104*, 6998−7002.
(36) Svab, Z.; Maliga, P. Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7003−7008.
(37) Maliga, P. Plastid Transformation in Higher Plants. *Annu. Rev. Plant Biol.* **2004**, *55*, 289−313.
(38) Gruissem, W.; Zurawskil, G. Identification and mutational analysis of the spinach chloroplast transfer RNA gene chloroplast DNA/RNA polymerase complexes (transcrip-tionally active chromosomes; Hallick et al for RNA polymerase activities present in recently developed chloroplast in vitro transcription systems. *Embo J.* **1985**, *4*, 1637−1644.
(39) Suzuki, J. Y.; Sriraman, P.; Svab, Z.; Maliga, P. Unique Architecture of the Plastid Ribosomal RNA Operon Promoter Recognized by the Multisubunit RNA Polymerase in Tobacco and Other Higher Plants. *Plant Cell* **2003**, *15*, 195−205.
(40) Orozco, E. M.; Mullet, J. E.; Hanley-Bowdoin, L.; Chua, N. H. In vitro transcription of chloroplast protein genes. *Methods Enzymol.* **1986**, *118*, 232−253.
(41) Bard, J.; Bourque, D. P.; Hildebrand, M.; Zaitlin, D. In vitro expression of chloroplast genes in lysates of higher plant chloroplasts. *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82*, 3983−3987.
(42) Bard, J. D. J.; Bourque, D. P.; Zaitlin, D. Coupled transcription-translation in chloroplast lysates. *Methods Enzymol.* **1986**, *118*, 270−282.
(43) Tao, K.-L. J.; Jagendorf, A. T. The ratio of free to membrane-bound chloroplast ribosomes. *Biochim. Biophys. Acta* **1973**, *324*, 518−532.

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

(44) Seigneurin-Berny, D.; Salvi, D.; Joyard, J.; Rolland, N. Purification of Intact Chloroplasts from Arabidopsis and Spinach Leaves by Isopycnic Centrifugation. *Curr. Protoc Cell Biol.* **2008**.
(45) Ho, J.; Theg, S. M.; Webber, A. The formation of stromules in vitro from chloroplasts isolated from Nicotiana benthamiana. *PLoS One* **2016**, *11*, 4−6.
(46) Hirose, T.; Sugiura, M. Cis-acting elements and trans-acting factors for accurate translation of chloroplast psbA mRNAs: development of an in vitro translation system from tobacco chloroplasts. *Embo J* **1996**, *15*, 1687−1695.
(47) Yukawa, M.; Kuroda, H.; Sugiura, M. A new in vitro translation system for non-radioactive assay from tobacco chloroplasts: Effect of pre-mRNA processing on translation in vitro. *Plant J.* **2007**, *49*, 367−376.
(48) Kim, M.; Mullet, J. E. Identification of a Sequence-Specific DNA Binding Factor Required for Transcription of the Barley Chloroplast Blue Light-Responsive psjbD-psbC Promoter. *Plant Cell* **1995**, *7*, 1445−1457.
(49) Kim, M.; Thum, K. E.; Morishige, D. T.; Mullet, J. E. Detailed architecture of the Barley chloroplast psbD-psbC blue light-responsive promoter. *J. Biol. Chem.* **1999**, *274*, 4684−4692.
(50) Kim, J.; Mullet, J. E. Ribosome-binding sites on chloroplast rbcL and psbA mRNAs and light-induced initiation of D1 translation. *Plant Mol. Biol.* **1994**, *25*, 437−448.
(51) Hirose, T.; Sugiura, M. Functional Shine-Dalgarno-Like Sequences for Translational Initiation of Chloroplast mRNAs. *Plant Cell Physiol.* **2004**, *45*, 114−117.
(52) Adachi, Y.; Kuroda, H.; Yukawa, Y.; Sugiura, M. Translation of partially overlapping psbD-psbC mRNAs in chloroplasts: the role of 5′-processing and translational coupling. *Nucleic Acids Res.* **2012**, *40*, 3152−3158.
(53) Suzuki, H.; Kuroda, H.; Yukawa, Y.; Sugiura, M. The downstream atpE cistron is efficiently translated via its own cis-element in partially overlapping atpB−atpE dicistronic mRNAs in chloroplasts. *Nucleic Acids Res.* **2011**, *39*, 9405−9412.
(54) Salis, H. M.; Mirsky, E. A.; Voigt, C. A. Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* **2009**, *27*, 946−950.
(55) Channarasappa, A. S.; Salis, H. M. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Res.* **2014**, *42*, 2646−2659.
(56) Salis, H. M. Translation Initiation is Controlled by RNA Folding Kinetics via a Ribosome Drafting Mechanism. *J. Am. Chem. Soc.* **2016**, *138*, 7016−7023.
(57) Borujeni, A. E.; et al. Precise quantification of translation inhibition by mRNA structures that overlap with the ribosomal footprint in N-terminal coding sequences. *Nucleic Acids Res.* **2017**, *45*, 5437−5448.
(58) Cetnar, D. P.; Salis, H. M. Systematic Quantification of Sequence and Structural Determinants Controlling mRNA stability in Bacterial Operons. *ACS Synth. Biol.* **2021**, *10*, 318−332.
(59) Reis, A. C.; Salis, H. M. An automated model test system for systematic development and improvement of gene expression models. *ACS Synth. Biol.* **2020**, *9*, 3145−3156.
(60) Kwon, Y. C.; Jewett, M. C. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. *Sci. Rep.* **2015**, *5*, 8663.
(61) Silverman, A. D.; Kelley-Loughnane, N.; Lucks, J. B.; Jewett, M. C. Deconstructing Cell-Free Extract Preparation for in Vitro Activation of Transcriptional Genetic Circuitry. *ACS Synth. Biol.* **2019**, *8*, 403−414.
(62) Baugh, C.; Grate, D.; Wilson, C. 2.8 Å crystal structure of the malachite green aptamer. *J. Mol. Biol.* **2000**, *301*, 117−128.
(63) Siegal-Gaskins, D.; Tuza, Z. A.; Kim, J.; Noireaux, V.; Murray, R. M. Gene circuit performance characterization and resource usage in a cell-free 'breadboard'. *ACS Synth. Biol.* **2014**, *3*, 416−425.
(64) Barkan, A. Expression of plastid genes: organelle-specific elaborations on a prokaryotic scaffold. *Plant Physiol.* **2011**, *155*, 1520−32.
(65) Lenzen, B.; et al. The Chloroplast RNA Binding Protein CP31A Has a Preference for mRNAs Encoding the Subunits of the Chloroplast NAD(P)H Dehydrogenase Complex and Is Required for Their Accumulation. *Int. J. Mol. Sci.* **2020**, *21*, 5633.
(66) Prikryl, J.; Rojas, M.; Schuster, G.; Barkan, A. Mechanism of RNA stabilization and translational activation by a pentatricopeptide repeat protein. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 415−420.
(67) Klaff, P.; Gruissem', W. Changes in Chloroplast mRNA Stability during Leaf Development. *Plant Cell* **1991**, *3*, 517−529.
(68) Bernstein, J. A.; Khodursky, A. B.; Lin, P.-H.; Lin-Chao, S.; Cohen, S. N. Global Analysis of MRNA Decay and Abundance in Escherichia Coli at Single-Gene Resolution Using Two-Color Fluorescent DNA Microarrays. *Biochem* **2002**, *99*, 9697−9702.
(69) Vieira, L. D. N.; et al. An improved protocol for intact chloroplasts and cpDNA isolation in conifers. *PLoS One* **2014**, *9*, No. e84792.
(70) Jewett, M. C.; Swartz, J. R. Mimicking the Escherichia coli Cytoplasmic Environment Activates Long-Lived and Efficient Cell-Free Protein Synthesis. *Biotechnol. Bioeng.* **2004**, *86*, 19−26.
(71) Record, M. T.; Courtenay, E. S.; Cayley, S.; Guttman, H. J. Responses of E. coli to osmotic stress: large changes in amounts of cytoplasmic solutes and water. *Trends Biochem. Sci.* **1998**, *23*, 143−148.
(72) Record, M. T.; Courtenay, E. S.; Cayley, S.; Guttman, H. J. Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. *Trends Biochem. Sci.* **1998**, *23*, 190−194.
(73) Schoborg, J. A.; Clark, L. G.; Choudhury, A.; Hodgman, C. E.; Jewett, M. C. Yeast knockout library allows for efficient testing of genomic mutations for cell-free protein synthesis. *Synth Syst. Biotechnol* **2016**, *1*, 2−6.
(74) White Tabor, C.; Tabor, H. Polyamines in Microorganisms. *Microbiol Rev.* **1985**, *49*, 81−99.
(75) Frugier, M.; Florentz, C.; Hosseini1, W.; Lehn2, J.-M.; Giegé, R. Synthetic polyamines stimulate in vitro transcription by T7 RNA polymerase. *Nucl Acids Res.* **1994**, *22*, 2784−2790.
(76) Iwata, M.; et al. T7 RNA polymerase activation and improvement of the transcriptional sequencing by polyamines. *Bioorg. Med. Chem.* **2000**, *8*, 2185−2194.
(77) Hong, S. H.; et al. Improving Cell-Free Protein Synthesis through Genome Engineering of Escherichia coli Lacking Release Factor 1. *ChemBiochem* **2015**, *16*, 844−853.
(78) Tohdoh, N.; Sugiura, M. The complete nucleotide sequence of a 16S ribosomal RNA gene from tobacco chloroplasts. *Gene* **1982**, *17*, 213−218.
(79) Buntru, M.; Vogel, S.; Stoff, K.; Spiegel, H.; Schillberg, S. A Versatile Coupled Cell-Free Transcription-Translation System Based on Tobacco BY-2 Cell Lysates. *Biotechnol. Bioeng.* **2015**, *112*, 867−878.
(80) Das Gupta, M.; et al. Scaling eukaryotic cell-free protein synthesis achieved with the versatile and high-yielding tobacco BY-2 cell lysate. *Biotechn. Bioeng.* **2023**, *120*, 2890−906.
(81) Chappell, J.; Jensen, K.; Freemont, P. S. Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Res.* **2013**, *41*, 3471−3481.
(82) Kelwick, R.; Webb, A. J.; MacDonald, J. T.; Freemont, P. S. Development of a Bacillus subtilis cell-free transcription-translation system for prototyping regulatory elements. *Metab. Eng.* **2016**, *38*, 370−381.
(83) Marshall, R.; Maxwell, C. S.; Collins, S. P.; Jacobsen, T.; Luo, M. L.; Begemann, M. B.; Gray, B. N.; January, E.; Singer, A.; He, Y.; Beisel, C. L.; et al. Rapid and Scalable Characterization of CRISPR Technologies Using an E. coli Cell-Free Transcription-Translation System. *Mol. Cell.* **2018**, *69*, 146−157.e3.

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411

(84) Swartz, J. R.; Jewet, M. C.; Woodrow, K. A. Cell-free protein synthesis with prokaryotic combined transcription-translation. In *Methods in Molecular Biology Second ed. Recombinant Gene Expression*,Balbás, P.; Lorence, A., Humana Press2004.
(85) Nakamura, M.; Sugiura, M. Selection of synonymous codons for better expression of recombinant proteins in tobacco chloroplasts. *Plant Biotechnol.* **2009**, *26*, 53−56.
(86) Gibson, D. G.; et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **2009**, *6*, 343−345.

https://doi.org/10.1021/acssynbio.4c00111
ACS Synth. Biol. 2024, 13, 2402−2411