Spheroplasted cells: a game changer for DNA delivery to diatoms

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ABSTRACT

Diatoms, vital to global carbon fixation and climate change mitigation, produce 20% of the world's fixed organic carbon annually. Their potential as cell factories for biofuels, proteins, and other high value chemicals remains underutilized due to a lack of genetic engineering tools, with DNA delivery being the biggest challenge. Here, we present an optimized, highly efficient electroporation method for delivering DNA constructs as large as 55.6 kb to *Phaeodactylum tricornutum*, a model diatom species and emerging chassis for algal biotechnology. We also demonstrate that with this optimized protocol, episomes can be assembled *de novo*, forgoing the need for time-consuming traditional cloning steps in *Escherichia coli* and *Saccharomyces cerevisiae*. By incorporating other technologies, such as CRISPR genome editing, this method will accelerate diatom-based synthetic biology projects and, therefore, the development of sustainable technologies. This method should also be applicable to other diatom species.

INTRODUCTION

Climate change, population growth, and soil depletion are among several challenges that existentially threaten current agricultural practices. Due to their comparatively rapid growth and higher photosynthetic efficiency, unicellular algae have been proposed as a sustainable agricultural alternative to traditional crops since the 1950s[1]. Diatoms (Bacillariophyta) are of particular interest for this purpose as they are widely distributed across the world's oceans[2], where they are one of the most photosynthetically productive taxa – accounting for an estimated 20% of total fixed organic carbon annually[3]. Despite their ubiquity and productivity, the use of diatoms as cell factories has been impeded by a lack of genetic engineering tools, with DNA delivery being one of the biggest impasses.

Phaeodactylum tricornutum is considered to be one of the most characterized model diatom species, having a fully sequenced telomere-to-telomere reference genome[4] in addition to molecular tools for DNA transformation[5–9], CRISPR-based genome engineering[10], protein expression[11], cloning of the whole mitochondrial[12] and chloroplast genomes[13], and more. These characteristics have made *P. tricornutum* an emerging chassis for diatom biotechnology[14] and the subject of a whole-genome synthesis effort[15]. The latter, termed the Pt-syn 1.0 project, has brought together an international consortium of phycologists with the aim of replacing all the native chromosomes in the nuclear, chloroplast, and mitochondrial genomes with synthetic counterparts. To actualize this, and to discover the full potential of diatoms as cell factories, a pipeline for efficiently and rapidly testing different DNA constructs in *P. tricornutum* must be established.

Existing methods for delivering DNA to *P. tricornutum* include biolistic bombardment[9], electroporation[6,8], polyethylene glycol (PEG) transformation[7], and bacterial conjugation[7]. Conjugation is the most efficient transformation method (4 transformants per 10^{-4} cells)[7], but also the most laborious to execute as it requires the creation and maintenance of a donor strain of *Escherichia coli*. This strain must contain a suitable broad-host-range conjugative plasmid in addition to the plasmid of interest. Complications can arise if these two plasmids share similar replication machinery, or if the plasmid of interest contains elements that are toxic when expressed in *E. coli*. The delivery of plasmids through electroporation and PEG transformation has been previously explored, but both methods demonstrated low efficiency (≤ 1 transformant per 10^{-8} cells) and high variability between experiments[7]. Electroporation is predominantly used for the integration of non-replicative DNA into the *P. tricornutum* nuclear genome, where efficiencies between 2.6 to 4.5 transformants per 10^5 cells have been previously reported[6,8]. We sought to optimize the electroporation method with the hopes of discovering a more rapid alternative to bacterial conjugation for the delivery of DNA to diatoms.

We found that enzymatic treatment of cells prior to electroporation increased transformation efficiency approximately 200-fold, with *P. tricornutum* cells grown on

agar plates demonstrating the highest overall transformation efficiency (~1 transformant per 10^{-4} cells). Past electroporation methods relied upon the use of 1 to 10 µg of DNA for transformation[6,8], but our optimized method yielded transformants when using as little as 1 ng of an 11 kb linear episome, with it being possible to deliver episomes as large as 55.6 kb. With this optimized method, it was also possible to electroporate multiple fragments at once, which were then assembled through non-homologous recombination in the algal nucleus to form a singular episome.

In this paper we describe an electroporation protocol for *P. tricornutum* that is faster and simpler to execute than bacterial conjugation, as well as more efficient than previous electroporation protocols. We also demonstrate that it is possible to assemble episomes directly in the algal cell, forgoing the need for traditional *E. coli* or *Saccharomyces cerevisiae* cloning steps. This protocol is suitable for rapid high throughput testing of several DNA constructs simultaneously and, due to its efficiency with low DNA amounts, can be used for the delivery of commercially synthesized episomes directly into the agal cell. This will be immensely valuable as the Pt-syn 1.0 project begins to build and test different synthetic chromosome designs. We anticipate that this protocol can be adapted to suit other diatom species.

RESULTS

Electroporation efficiency increases with enzymatic treatment of cells

Our initial attempts at electroporation using a previously described protocol[16] that was adapted from the methods of Zhang and Hu[6] yielded no transformants. After several failed attempts, we discovered that adjusting the capacitance from 25 to 50 µF during electroporation yielded transformants for both plasmids and marker cassettes, though efficiency was highly variable between experiments (Supplementary Table S1, Exp. 1 to 81). We used this method to deliver an 11 kb PCR-amplified plasmid, pPtGE31_ΔPtR, to see if the construct would integrate into the genome or be replicated as a linear episome in *P. tricornutum*. To our surprise, all analysed transformants demonstrated that the linear episome had been circularized in the algal nucleus through non-homologous end joining (NHEJ).

When this electroporation protocol was repeated using cells that had reached the late stationary phase of growth, we obtained up to 272 transformants per 10^8 cells with the same 11 kb PCR-amplified episome (Supplementary Table S1, Exp. 82 to 89). This represented a nearly 20-fold increase in efficiency when compared to transformations with early-log phase cultures (Exp. 80 and 81). Stationary phase cultures demonstrated noticeable differences in cell phenotype when compared to early-log phase cultures, with there being a higher abundance of oval-morphotype cells and protoplasts (i.e., diatoms lacking a cell wall) in the older cultures. *P. tricornutum* cells can appear in three different morphotypes – fusiform, oval, and triradiate – and can switch morphotypes in response to environmental stimuli[17,18]. This

led us to hypothesize that morphotype and cell wall integrity were integral to electroporation transformation efficiency.

To test this hypothesis, we spheroplasted early-log phase *P. tricornutum* cells harvested from agar plates. Our previous experiments had used early log phase liquid cultures, which predominantly consisted of the fusiform morphotype; in past experiments, we had noticed a higher abundance of the oval morphotype in solid cultures harvested at the same growth stage. Plate-derived cells were spheroplasted using the enzyme alcalase, a serine protease, which has been previously shown to degrade the proteinaceous components of the *P. tricornutum* cell wall[19]. We observed that 100 µl of alcalase at an activity of approximately 3 Anson units per ml (i.e., 300 mAnson units) was enough to fully protoplast 3 x 10^8 plate-derived cells (Fig. 1A and B), which then became very fragile and prone to rupture from osmotic pressure or physical damage. Treatment of 3 x 10^8 cells with 10 µl of alcalase (30 mAnson units) was enough to protoplast some cells, whilst leaving others with partially degraded cell walls (i.e., spheroplasts).

Plate-derived cells (3 x 10^8) were treated with 0.3 mAnson units, 3 mAnson units, and 30 mAnson units of alcalase prior to the washing steps of electroporation. Transformation efficiency increased in all cases (Fig. 1C) when electroporating the same 11 kb PCR-amplified episome as before (Fig. 1D), with the 3 mAnson treatment demonstrating the highest increase in efficiency. Based on these results, we went forward with using 1 mAnson unit of alcalase per 10^8 cells when spheroplasting *P. tricornutum* for electroporation.

Electroporation efficiency of cells grown in liquid and solid media

We tested the electroporation efficiency of *P. tricornutum* cells grown in liquid media and on agar plates, with and without alcalase treatment, across five biological replicates (Fig. 1E) using the same PCR-amplified 11-kb episome as before. Though transformation efficiency varied between experiments, it was evident that the plate-derived alcalase-treated cells were drastically more efficient (~1.2 transformants per 10^4 cells) than untreated plate-derived cells (~6.0 transformants per 10^7 cells), untreated liquid-derived cells (~4.2 transformants per 10^7 cells), and treated liquid-derived cells (~1.6 transformants per 10^6 cells, Supplementary Table S2). Based on this result, all proceeding electroporations were conducted with cells derived from agar plates and spheroplasted with 1 mAnson unit of alcalase per 10^8 cells.

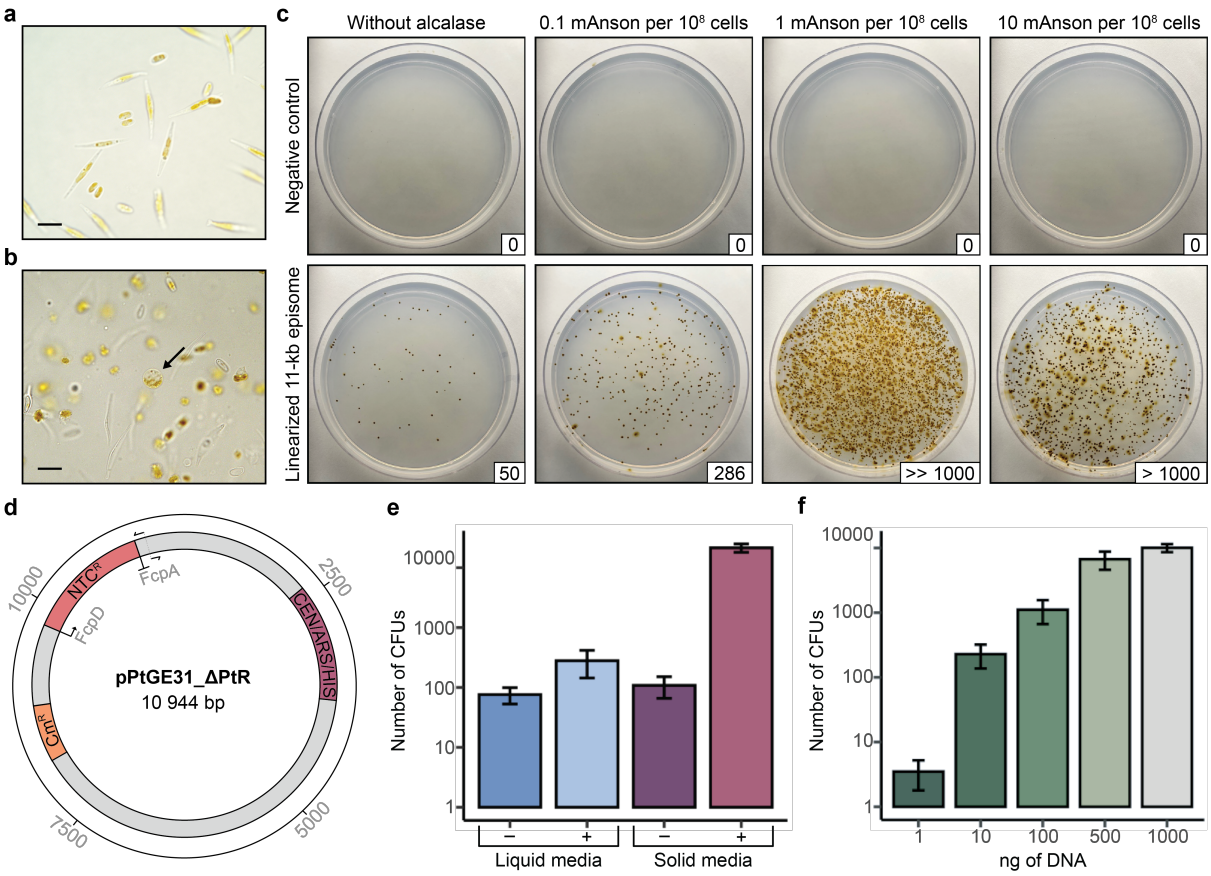

Figure 1. Spheroplasting *P. tricornutum* cells increases transformation efficiency. (**a**) Plate-derived *P. tricornutum* cells pre- and (**b**) post-treatment with 100 mAnson units of alcalase per 10^8 cells. The arrow indicates a completely protoplasted cell, and the scale bars represents 10 µm. (**c**) Comparison of agar plate derived *P. tricornutum* cells treated with differing amounts of alcalase prior to electroporation. Cells were either electroporated with no DNA (negative control) or the 11 kb episome pPtGE31_ΔPtR, which was obtained through PCR amplification. The number of colony forming units (CFUs) is depicted in the bottom right corner for each transformation plate. Following electroporation, half of the total reaction was plated on ¼-salt L1 plates supplemented with 100 µg/ml nourseothricin. (**d**) A plasmid map of pPtGE31_ΔPtR, the episome used for transformations in panels c, e, and f. Primers depict where the plasmid was linearized during PCR-amplification. This plasmid contains a yeast centromere (CEN) and origin of replication (ARS) sequence, which enables episome replication and stability in *P. tricornutum*[7], as well as a bacterial chloramphenicol resistance marker (Cm^R) and an algal nourseothricin resistance marker (Ntc^R) driven by the FcpD/FcpA promoter-terminator pair[10]. (**e**) A comparison of transformation efficiency for cells cultured in liquid or solid media with (+) or without (-) alcalase treatment prior to electroporation. (**f**) The number of *P. tricornutum* CFUs obtained with different nanograms of DNA during electroporation. Error bars denote the standard error of the mean from at least four biological replicates per experiment.

Transformation efficiency with varying amounts of DNA

Our prior transformations were all conducted with ~500 ng of a PCR-amplified 11-kb plasmid, pPtGE31_ ΔPtR (Fig. 1D). We were interested to see how transformation efficiency varied when using differing amounts of this construct, and most importantly, what the minimum amount of DNA for transformation is. We electroporated as little as 1 ng and up to 1 µg of PCR-amplified pPtGE31_ ΔPtR across four biological replicates (Fig. 1F, data summarized in Supplementary Table S3). In all but one experiment, we were able to recover transformants when using just 1 ng of DNA during electroporation. Increasing the amount of DNA to 10 ng routinely gave rise to hundreds transformants, with there being an average of 372 CFUs per reaction. Interestingly, there was only a small change in transformation efficiency when the amount of DNA was doubled from 500 ng to 1 µg, suggesting that 500 ng of linear pPtGE31_ ΔPtR was enough to nearly saturate the electroporation reaction.

Transforming linear and circular episomes to the algal nucleus

Our past explorations had relied upon the use of linear PCR-amplified DNA with blunt, non-phosphorylated termini. To test the efficiency of different structural forms of DNA, we prepared other variations of pPtGE31_ ΔPtR (Fig. 2A). The termini of the linear constructs varied in position along the episome and/or the properties of the termini (i.e., blunt or sticky ends, with or without 5′ phosphorylation). These constructs were adjusted to be equimolar prior to conducting electroporation to *P. tricornutum*. We found that there were nearly double the amount of CFUs when transforming any structural form of linear DNA compared to the circular episome (Fig. 2B).

Our early explorations with electroporation had demonstrated that linear pPtGE31_ ΔPtR gets circularized in the *P. tricornutum* nucleus, but we had not yet performed comprehensive screening to determine if any indels were accruing during this NHEJ repair process. To explore what occurs during termini fusion, ten colonies were repatched from the transformations with circular and PCR-amplified pPtGE31_ ΔPtR for additional screening. After two repatches, DNA was isolated from the transformants and analysed using a set of primers that spans where the termini are expected to recombine for the PCR-amplified construct, as well as a region of the episome backbone (Fig. 2C). Colonies transformed with circular DNA did not demonstrate any unexpected insertions or deletions in the screened regions (Fig 2D), as expected. Whole plasmid sequencing of circular colonies 1 and 2 confirmed that the plasmids had not accrued any mutations or indels during transformation.

Colonies transformed with PCR-amplified DNA appeared to have indels in the region where termini fusion occurs (Fig. 2E). We conducted Sanger sequencing of the termini region for colonies 1, 2, and 6 to 10, which confirmed that indels of varying sizes had accrued in this region for every colony screened (Supplementary Fig. S1).

Whole plasmid sequencing of linear colonies 1 and 8 further confirmed that indels had accrued in the termini region where the episome was being fused together, whereas all other regions of the episome had remained unchanged. Interestingly, the ~500-bp insertions in colonies 2, 8, and 10 were not complimentary to any regions of the episome or algal genome. When these insertions were queried using BLASTn, they aligned to various regions of the salmon and/or trout genome accessions.

Single stranded salmon sperm (ssss) DNA is used as a carrier DNA during electroporation, and to our surprise, we had discovered that it could be captured during NHEJ of the episome termini. We attempted electroporation without ssssDNA to see if this would impact transformation efficiency, and it resulted in a 15-fold reduction in the number of CFUs (Supplementary Fig. S2), highlighting its importance as an additive for transformation.

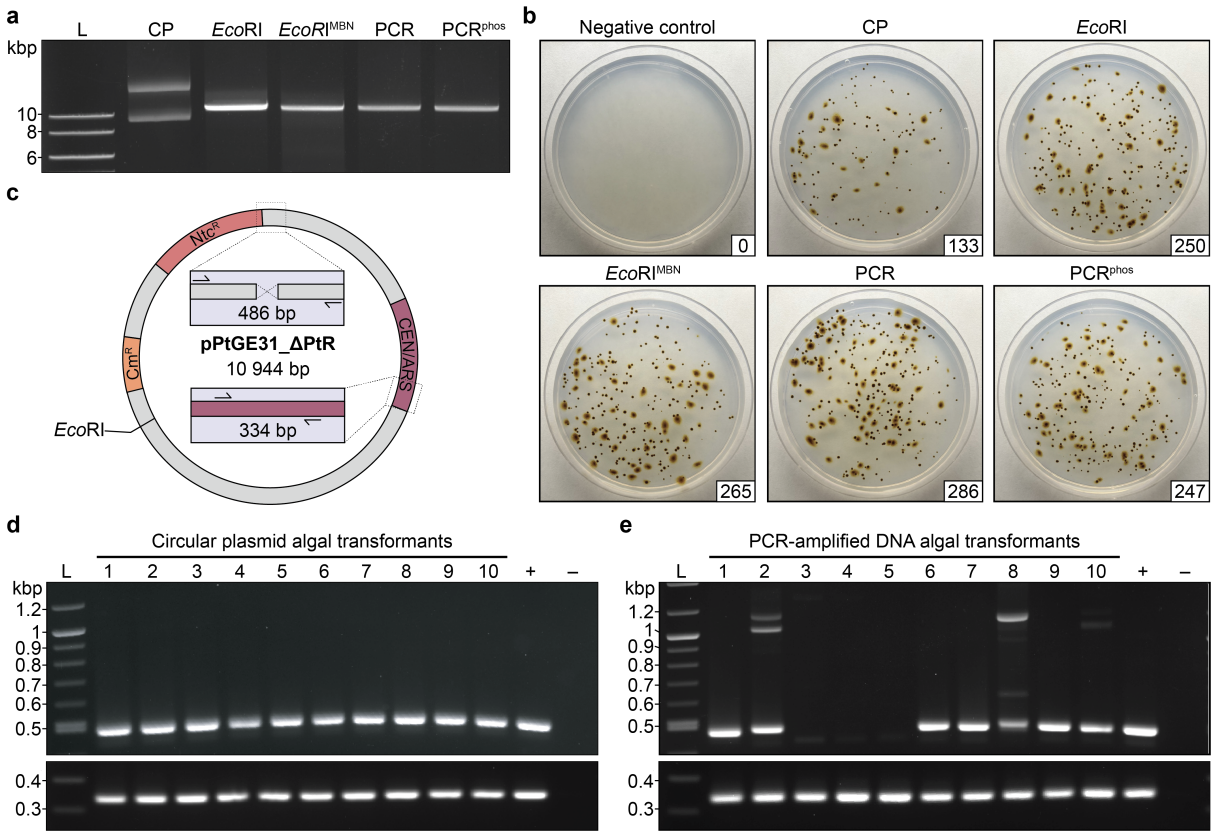

Figure 2. Comparing the transformation efficiency of linear, circularized, and phosphorylated DNA. (**a**) An agarose gel depicting different preparations of the plasmid pPtGE31_ ΔPtR. CP: circular plasmid, *Eco*RI: restriction enzyme digested plasmid, *Eco*RI^{MBN}: restriction enzyme digested plasmid with additional mung bean nuclease treatment to remove sticky ends, PCR: PCR-amplified linear plasmid DNA, and PCR^{phos}: PCR-amplified linear plasmid DNA generated with phosphorylated primers. (**b**) Comparison of *P. tricornutum* transformants electroporated with different preparations of pPtGE31_ ΔPtR. Following electroporation, one-tenth of the total reaction was plated on ¼-salt L1 plates supplemented with 100 µg/ml nourseothricin. Plates are labelled according to the type of DNA that was transformed, as described

above. The number of CFUs is shown in the bottom right corner for each plate. (**c**) A plasmid map depicting the regions that were screened when assessing passaged algal transformants. The 486 bp junction spans the region where pPtGE31_ ΔPtR was split for PCR amplification, whereas the 334 bp junction spans a region of the plasmid backbone. (**d**) Screening 10 algal colonies that had been transformed with circular pPtGE31_ ΔPtR or (**e**) PCR-amplified pPtGE31_ ΔPtR. Dilute pPtGE31_ ΔPtR and genomic *P. tricornutum* DNA were used for the positive and negative controls, respectively.

Electroporation and recovery of a large plasmid

It has been demonstrated in other organisms that as episome size increases, electroporation efficiency decreases[20,21]. We sought to test the limits of electroporation in *P. tricornutum* by attempting transformation with pSC5, a 55.6 kb broad-host-range conjugative episome[22] (Fig. 3C, Addgene ID: 188602). This episome was chosen as it had been previously transformed into *P. tricornutum* through bacterial conjugation, where it was demonstrated to be stable and recoverable.

Equimolar amounts of circular and linear pSC5 were prepared along with PCR-amplified pPtGE31_ ΔPtR (Fig. 3A). Electroporation with either form of pSC5 gave rise to transformants, though the number of CFUs was orders of magnitude lower than electroporation with a linear 11 kb episome (Fig. 3B), as anticipated. Transformation efficiency with linear pSC5 was three times greater than with the circular form when averaged across three biological replicates (Supplementary Table S4), with there being an average of 12 CFUs per reaction.

To determine if the complete 55.6 kb episome had been successfully electroporated into *P. tricornutum*, we passaged four colonies from each of the transformations with circular and linear pSC5. DNA was isolated from the algal colonies and electroporated into *E. coli*. Then, we performed conjugation using a pool of *E. coli* colonies for each transformation (Fig. 3D). Here, if the transformants contain an intact version of pSC5, the episome will facilitate conjugation and self-mobilization into a recipient strain harbouring pSAP[13] (Addgene ID: 206429), a 10 kb plasmid that contains a chloramphenicol resistance marker. Successful transconjugants will harbour pSC5 and pSAP, therefore carrying resistance to both gentamycin and chloramphenicol. Conjugation proved successful for seven of the eight *E. coli* pools, demonstrating that intact pSC5 had been successfully electroporated and maintained in the majority of *P. tricornutum* transformants tested (Fig. 3E).

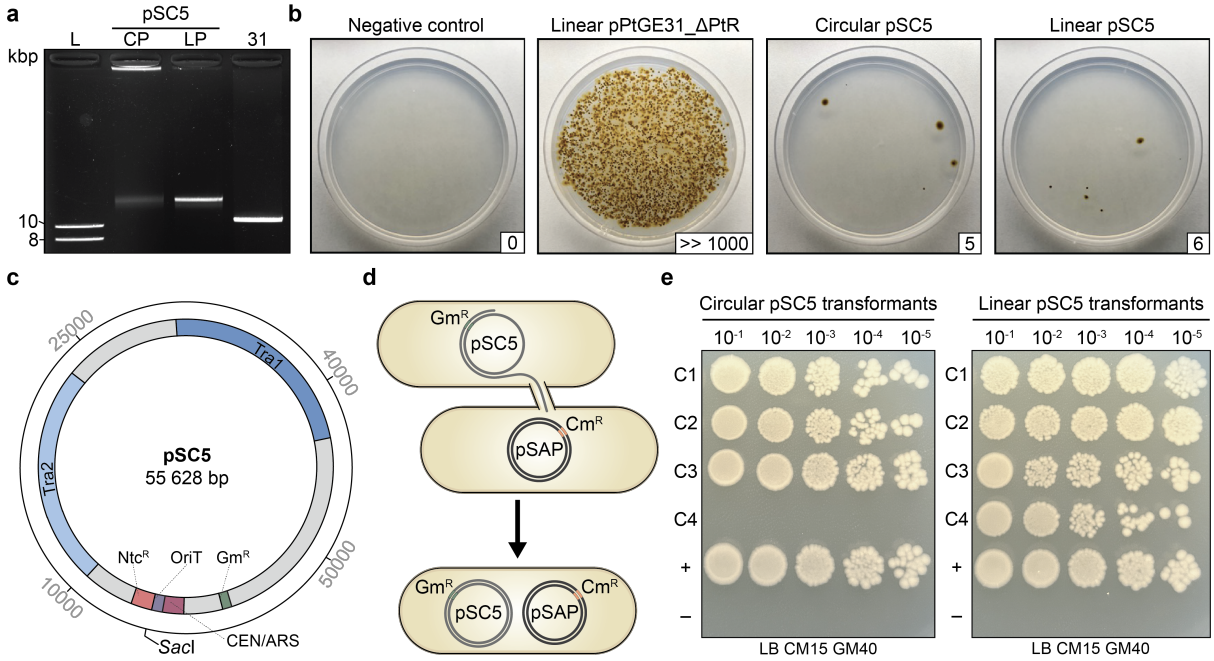

Figure 3. Electroporation and recovery of a 55.6 kb conjugative plasmid. (**a**) An agarose gel depicting the circular pSC5 (CP), linearized pSC5 (LP), and PCR-amplified pPtGE31_ ΔPtR (31). (**b**) Electroporation of circular or linear variants of pSC5 and PCR-amplified pPtGE31_ ΔPtR into *P. tricornutum* cells. Following electroporation, half of the total reaction was plated on ¼-salt L1 plates supplemented with 100 µg/ml nourseothricin. Plates are labelled according to the type of DNA that was transformed, as described above. The number of CFUs is shown in the bottom right corner for each plate. (**c**) A plasmid map of pSC5. The transfer regions (Tra1 and Tra2) encode for essential conjugative proteins. The origin of transfer (OriT) serves as a recognition sequence for the conjugative protein apparatus, facilitating the transfer of pSC5 during conjugation. The plasmid contains selection markers for bacteria (Gm^R, gentamycin resistance) and *P. tricornutum* (Ntc^R, nourseothricin resistance) as well as a yeast centromere (CEN) and replication origin (ARS), which enable episome stability and replication in *P. tricornutum*. (**d**) A donor strain of *E. coli* harbouring pSC5, which confers gentamycin resistance, conjugating to a recipient strain of *E. coli* harbouring pSAP, which confers chloramphenicol resistance. Successful conjugation leads to the creation of a transconjugant strain that is gentamycin and chloramphenicol resistant. (**e**) Testing the conjugation efficiency of *E. coli* strains harbouring episomes that had been recovered from *P. tricornutum* transformants electroporated with circular or linear pSC5. Strains harbouring pSC5 or pSAP were used as the positive and negative controls, respectively. Dilutions of 10^{-1} to 10^{-5} were spot plated on LB plates supplemented with 15 µg/ml chloramphenicol and 40 µg/ml gentamicin.

Electroporation and recombination of multiple DNA fragments

The surprising discovery of salmon DNA inserted into the termini region of PCR-amplified pPtGE31_ΔPtR led us to hypothesize that *P. tricornutum* could assemble

multiple DNA fragments into a single episome through NHEJ. To test this, we created an episome, pPtGE31_ShBle, that contains the NAT and ShBle markers conferring resistance to nourseothricin and zeocin, respectively. The episome pPtGE31_ShBle was then amplified as two non-overlapping fragments, each containing one of the algal selective markers. The fragments were adjusted to be equimolar prior to electroporation into *P. tricornutum* (Fig. 4A).

Cells transformed with both fragments simultaneously were plated on nourseothricin- or zeocin-containing plates initially (Supplementary Fig. S3). Out of the 116 colonies passaged post-electroporation, 94 demonstrated double resistance to both nourseothricin and zeocin (Supplementary Table S5). Twenty transformants, ten derived from each initial transformation plate, were passaged onto double-selection media, then DNA isolation and PCR screens were performed. These colonies are denoted with an N- or Z- to depict which initial selection plate they were derived from (N: nourseothricin, Z: zeocin).

Two orientations are expected when the fragments amplified from pPtGE31_ShBle are re-assembled. We designed a PCR screen that would not only assess if both fragments had been recombined, but could also determine the orientation of the fragments (Fig. 4B). When screening algal transformants, we only used the primer set spanning junction B as junction A contains primers that bind to the FcpD promoter and FcpA terminator, which are present in the episome and algal genome. Of the twenty colonies screened, nine had amplification across junction B, with only five colonies demonstrating the expected amplicon sizes (Supplementary Fig. S4). The other four colonies either demonstrated multiple amplicons, some of which were the expected sizes, and/or amplicons that were larger than expected. Based on this initial screen, DNA from colonies N-C6, N-C9, N-C10, Z-C2, Z-C4, and Z-C5 were electroporated into *E. coli* for further screening.

Three *E. coli* transformants were assessed per algal colony using the primer sets spanning junctions A and B (Fig. 4C and D). Interestingly, the *E. coli* transformants derived from algal colonies N-C6, N-C10, and Z-C2 demonstrated different banding patterns, suggesting that the initial algal colony likely had a pool of assembled episomes. All *E. coli* transformants from algal colony N-C9 depicted the expected banding pattern for orientation 2, whereas all *E. coli* transformants from algal colony Z-C5 depicted the expected banding pattern for orientation 1. DNA from *E. coli* colonies N-C6-E1/E2, N-C9-E1, N-C10-E1/E2, Z-C2-E1/E2/E2, Z-C4-E1, and Z-C5-E1 were sent for whole-plasmid sequencing to validate if assembly had occurred.

The sequencing results demonstrated that recombination of fragments 1 and 2 had occurred in all assessed *P. tricornutum* transformants (Supplementary Fig. S5). In the majority of assembled episomes, multiple partial copies of fragment 1 and 2 had been recombined. One extreme case was *E. coli* colony Z-C2-E2, which harboured a 41.5 kb episome containing eight partial or whole copies of both fragments 1 and 2. Only *E. coli* colonies N-C9-E1, N-C10-E1, Z-C2-E3, and Z-C5-E1 contained the

expected assembled episomes. Sequencing of *E. coli* transformants derived from the same algal colony revealed that algal colonies N-C6, N-C10, and Z-C2 possessed a pool of assembled episomes, as we had hypothesized.

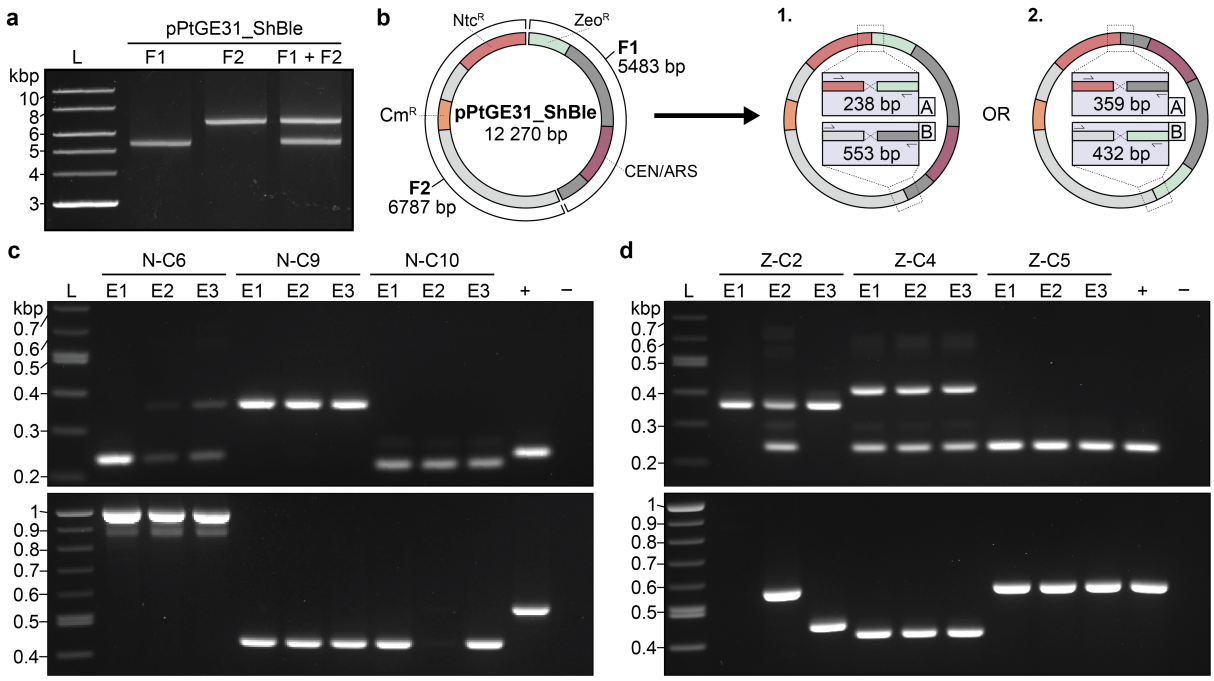

Figure 4. Electroporation and assembly of two non-overlapping fragments in *P. tricornutum*. (**a**) The episome pPtGE31_ShBle was amplified as two fragments (F1: fragment 1, F2: fragment 2). The fragments were adjusted to be equimolar before combining them into an assembly mixture (F1 + F2). (**b**) A plasmid map of pPtGE31_ShBle and a depiction of the different possible orientations post-assembly. The episome contains algal selection markers for nourseothricin (Ntc^R) and zeocin (Zeo^R), a bacterial selection marker for chloramphenicol (Cm^R), as well as a yeast centromere (CEN) and replication origin (ARS), which enable episome stability and replication in *P. tricornutum*. F1 and F2 do not share any overlapping sequences, and thus can assemble in two different orientations. Primer sets were designed to span the two junctions, A and B, where recombination is expected to occur. The amplicons will vary in size depending on the orientation of the fragments. (**c**) Screening three *E. coli* transformants per algal colony using primers spanning junctions A and B. DNA were isolated from algal transformants that were electroporated with F1 + F2 and selected initially with nourseothricin or (**d**) zeocin. Dilute pPtGE31_ ShBle and water were used for the positive and negative controls, respectively.

DISCUSSION

Our initial attempts at conducting electroporation using previously described methods[6,16] proved unsuccessful until we adjusted the capacitance parameter from 25

to 50 μF. We suspect that variation between equipment, reagents, culture growth conditions and more could have led to this discrepancy. With this adjustment, we were able to electroporate a linearized 11 kb episome into *P. tricornutum*. Analysis of algal transformants demonstrated that the linear episome had been circularized and maintained extra-chromosomally in the algal nucleus. With this discovery, we sought to explore electroporation as an alternative delivery strategy for establishing episomes in *P. tricornutum*.

Past electroporation protocols[6,16] recommend the use of *P. tricornutum* cells cultured in liquid media and harvested during the early-log phase of growth, however, we found that stationary-phase liquid cultures were up to 20-fold more efficient at transformation than early-log phase cultures. We used light microscopy to visually compare early-log phase cells to stationary phase cells, revealing key morphological differences between the two that suggested pleomorphic changes and cell wall degradation were influential to transformation.

We tested the transformation efficiency of early-log phase cells derived from agar plates subjected to varying intensities of spheroplasting. Spheroplasting with 0.1 to 1 mAnson unit of alcalase per 10^8 cells drastically improved transformation efficiency (Fig. 1C), demonstrating the effect of the cell wall on electroporation. When we compared early-log phase cells derived from liquid or solid cultures, with or without spheroplasting treatment, it was evident that the spheroplasted agar plate-derived cultures were the most efficient (~76- to 280-fold increase in transformation efficiency, Supplementary Table S2). This may be due to differences in the cell wall composition between cells grown in liquid cultures, which predominantly consist of the fusiform morphotype, and solid cultures, which demonstrate a higher abundance of the oval cell morphotype. Alternatively, there may be other pleomorphic changes associated with growth on solid media that enable agar plate-derived cultures to acclimate better post-transformation, as selection takes place on agar plates. The latter hypothesis is supported by the fact that similar amounts of fusiform and oval colonies are apparent on the transformation plates post-electroporation; if the oval morphotype was more conducive to transformation, we would expect most transformants to retain this morphotype post-electroporation (Supplementary Fig. S6). A similar phenomena occurs during conjugation, where agar plate-derived *P. tricornutum* cells are several times more efficient than cells derived from liquid cultures[7].

With this optimized protocol, it was then possible to answer unresolved questions and test the limits of electroporation to *P. tricornutum*. We demonstrated that linear episomes are more efficient for transformation than circular episomes, but indels are likely to accrue during NHEJ of the episome termini. It was possible to recover transformants when using as little as 1 ng of an linear 11 kb episome, a stark contrast to existing protocols that call for the use of 1 to 10 μg of DNA[6,8]. This presents the opportunity to go directly from the synthesis of an episome to transformation in *P. tricornutum* as it is no longer necessary to isolate large quantities of DNA from an intermediate host like *E. coli*. This method was then used to successfully deliver and

recover a 55.6 kb conjugative episome, though transformation efficiency decreased drastically when electroporating an episome of this size. Perhaps most interesting, we demonstrated that it is possible to deliver and assemble multiple fragments in *P. tricornutum* through NHEJ. This presents the opportunity to amplify fragments from disparate episomes and assemble them directly in *P. tricornutum*, foregoing the need for traditional cloning in *E. coli* and *S. cerevisiae*.

Our optimized electroporation method demonstrated an efficiency of ~1 transformant per 10^4 cells when electroporating an 11 kb linear episome, which is comparable to the transformation efficiency for bacterial conjugation[7] (4 transformants per 10^4 cells). Episome sizes smaller than 11 kb were not tested, however, we expect that electroporation efficiency would be higher in these cases. Electroporation offers a more facile and rapid method for testing episomes when compared to conjugation (Fig. 5A), as the latter requires the creation of a donor strain of *E. coli* that contains a suitable broad-host-range conjugative plasmid in addition to the episome of interest (Fig. 5B). Establishing the donor strain is a time-consuming process that often requires intensive screening to ensure that the intact constructs are present, unchanged, and stably maintained prior to conjugation. Furthermore, additional screening is required post-conjugation to ensure the episome of interest was intactly transferred to the algal cell as rearrangements, mutations, and deletions can occur during this process[7,23]. Electroporation forgoes the need for a donor *E. coli* strain entirely, and resultantly, offers a more simple and rapid pipeline for delivering and testing episomes in the algal cell.

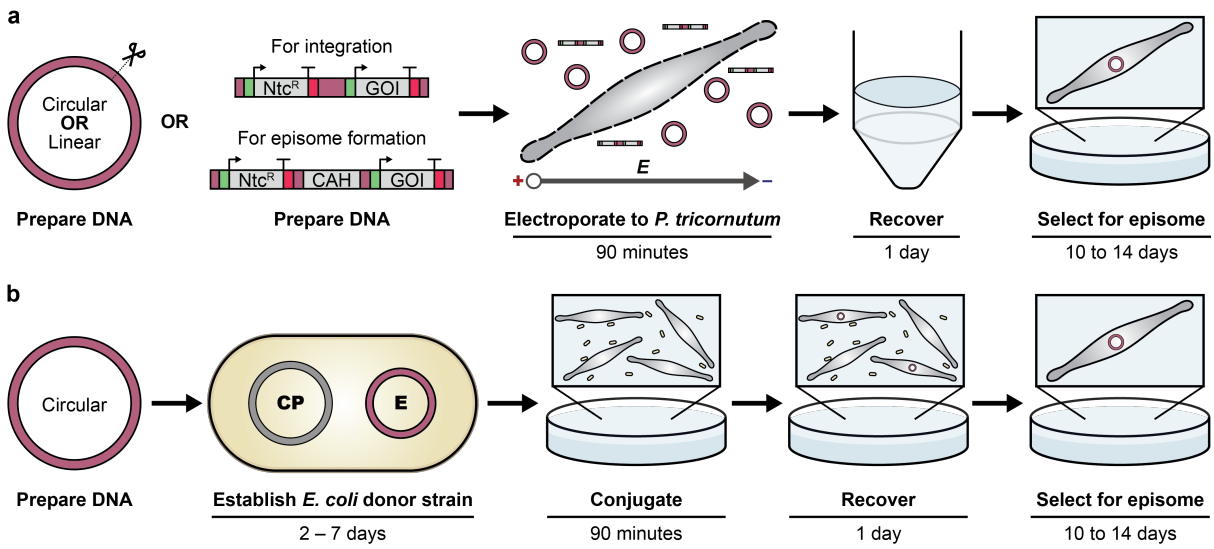

Figure 5. A comparison of electroporation and bacterial conjugation as methods for transforming DNA into *P. tricornutum*. (**a**) Circular or linear episomes, integrative cassettes, or PCR-amplified episomes can be electroporated into *P. tricornutum* (Ntc^R: nourseothricin resistance marker, GOI: gene of interest, CAH: yeast centromere, autonomously replicating sequence, and histidine auxotrophic marker).

Electroporation takes approximately 90 minutes to conduct, after which the cells are recovered in liquid media for up to 1 day. The culture is transferred to a selection plate, with transformants appearing after 10 to 14 days. (**b**) A circular episome (E) is installed in an *E. coli* strain harbouring a conjugative plasmid (CP) to create a donor strain. This process can be time consuming as it is necessary to ensure that E is stably maintained alongside CP. The donor strain and *P. tricornutum* are conjugated for 90 minutes, after which the conjugation plates are moved to an algal growth chamber to recover for at least 1 day. The culture is then transferred to a selection plate, with transconjugants appearing after 10 to 14 days.

Going forward, it will be interesting to test the spheroplasting electroporation method in other diatom species and with alternative transformation apparatuses. The bacterial conjugation transformation method was first developed for *P. tricornutum*[7], but then quickly adopted for *Thalassiosira pseudonana*[7], *Fistulifera solaris*[24], *Nitzschia captiva*[25], and *Nannochloropsis oceanica*[26]. We anticipate that our optimized electroporation method could be applied to other diatom species as well, though the exact spheroplasting conditions may need to be optimized on a case-by-case basis. Likewise, we anticipate that spheroplasted *P. tricornutum* cells will be more conducive to DNA uptake when using a multi-pulse electroporator or a biolistic gene gun. Multi-pulse electroporators are less common than conventional single-pulse electroporators, but are thought to be more effective at transformation in various algal and plant species. Past electroporation protocols demonstrated that multi-pulse electroporation[8] was approximately two times more efficient than single-pulse electroporation[6] when transforming *P. tricornutum* (4.5 transformants per 10^5 cells and 2.8 transformants per 10^5 cells, respectively). Though electroporation and biolistic transformation vastly differ in terms of praxis, in theory, both rely on the brute physical movement of DNA into cells. The first physical barrier to entry is the cell wall, so it follows that spheroplasting may enhance DNA uptake for biolistic bombardment as well.

Our ultimate objective with the Pt-syn 1.0 project is to replace all native chromosomes in the nuclear, chloroplast, and mitochondrial genomes with synthetic counterparts[15]. A necessary first step towards this goal is to establish methods for building and establishing synthetic chromosomes in the algal cell. Our optimized electroporation method provides a rapid method to not only deliver and test various synthetic constructs in the algal nucleus, but also provides a method to assemble *de novo* constructs through NHEJ. It was demonstrated that when transforming two non-homologous fragments, upwards of ten fragments could recombine to form a hybrid episome, and multiple assembled episomes could be recovered from a single transformant. In the future, it will be necessary to improve the efficiency of this assembly method to make it more practical and reliable. Splitting the fragments in the open reading frame(s) of a selectable marker(s) could increase assembly efficiency as it would make only certain fragment combinations possible. Alternatively, it may be

possible to assemble fragments with overlapping sequences through homologous recombination (HR) in *P. tricornutum*. HR-directed assembly is much more efficient than NHEJ assembly in organisms like *S. cerevisiae*, but this has yet to be explored in *P. tricornutum*.

To conclude, this article presents an optimized electroporation method for high efficiency transformation of *P. tricornutum*, an emerging diatom chassis for algal synthetic biology (Supplementary Fig. S7). This method can be used to directly transform commercially synthesized constructs and to assemble episomes *de novo*, forgoing the need for traditional cloning and DNA isolation methods with intermediate hosts. In the future, it will be worthwhile to test this spheroplasting method in other diatom species and to use this technique to improve other transformation techniques. Our electroporation method will be immensely valuable as the Pt-syn 1.0 project begins to build and test different synthetic chromosome designs.

METHODS

Microbial strains and growth conditions

P. tricornutum (Culture Collection of Algae, the University of Texas at Austin [UTEX], catalog number: 646) was cultured in liquid L1 media or on ½-salt L1 1% agar plates (w/v) in a growth chamber set to 18 ºC and a light/dark cycle of 16/8 hours. Cultures were irradiated with white LED lights (Mars Hydro, model: TSL 2000) set to photon flux densities of ~75 µE m−2 s−1 (liquid cultures) or ~55 µE m−2 s−1 (agar plate cultures). Agar plates were placed in clear polystyrene trays to reduce desiccation. L1 media was prepared as previously described and lacks silica[7].

S. cerevisiae strain VL6-48 (American Type Culture Collection [ATCC], catalog number: MYA-3666) was grown in 2X YPAD media or plated on complete minimal media lacking histidine with 1% agar (w/v) and 1 M D-sorbitol. Yeast cultures were incubated at 30 ºC, with liquid cultures placed on a shaker set to 225 rpm. Agar plates were sealed with parafilm to reduce desiccation. Yeast media were prepared as previously described[27].

E. coli strain EPI300 (LGC Biosearch Technologies, Lucigen, catalog number: EC300110) was grown in lysogeny broth (Miller formula) with or without 1% agar (w/v) and supplemented with 15 µg/ml chloramphenicol and/or 40 µg/ml gentamycin and/or 100 µg/ml L-arabinose. Cultures were placed in an incubator set to 37 ºC, with liquid cultures placed on a shaker set to 225 rotations per minute (rpm).

PCR amplification

Episomes and/or fragments for transformation and/or assembly were amplified with GXL polymerase (Takara, catalog number: R050A) using the rapid PCR protocol (all

primers are listed in Supplementary Table S6). Of note, we used both phosphorylated and non-phosphorylated primers when amplifying pPtGE31_ ΔPtR as a linear episome. PCR reactions were conducted with a total of 25 to 35 cycles depending on amplification efficiency. Fragments that were amplified from an episome template were treated with 10 units (0.5 µl) of *Dpn*I (New England Biolabs, catalog number: R0176) and incubated at 37 °C for 30 min before deactivation at 80 °C for 20 min. After deactivation, fragments were column-purified using the PureLink PCR Purification Kit (Invitrogen, catalog number: K310001) and eluted with ddH_2O.

PCR screening of algal and E. coli transformants

DNA isolated from algal and *E. coli* transformants was screened for the presence of specific episomal regions using a PCR assay. Here, 1 µl of DNA isolated through alkaline lysis was used as template in a 10 µl SuperPlex PCR reaction (Takara, catalog number: 638543) with a single primer set at a time (all primers are listed in Supplementary Table S6). Reactions were carried out according to the manufacturer guidelines for a total of 30 cycles.

DNA isolation

Episomes were isolated from *P. tricornutum*, *S. cerevisiae*, and *E. coli* using a modified alkaline lysis protocol that was previously described in Karas et al[7]. We modified steps 1 to 3 for *P. tricornutum* DNA isolation, which is described below.

Steps 1 to 3 for *P. tricornutum*: (**1**) *P. tricornutum* transformants that were repatched at least twice were grown to high density on a ¼-salt L1 plates supplemented with the appropriate antibiotic(s) (i.e., 100 µg/ml nourseothricin or zeocin for single selection, double selection plates contained both antibiotics at 50 µg/ml). Colonies were passaged as large streaks to ensure there were enough cells for DNA isolation – typically, a single plate would be used to passage 6 colonies that were struck out to each cover approximately a sixth of the plate. Plates were grown until a reasonable cell density was achieved, which typically took 4 days for nourseothricin plates and upwards of 6 days for zeocin or double-selection plates. (**2**) Cells were scraped and resuspend in 250 µl of resuspension buffer, which consisted of 245 µl of P1 (Qiagen, catalog number: 19051) and 5 µl of alcalase (Sigma-Aldrich, catalog number: 126741). (**3**) The resuspended cell mixture was vortexed for 1 to 3 seconds, then placed in a heating block set to 56 ºC for 10 minutes – this is within the optimal temperature range for alcalase activity, according to the manufacturer. After 10 minutes passed, the cells were vortexed again for 1 to 3 seconds to ensure there were no cell clumps.

For *E. coli*-derived episomes smaller than 11 kb, we used column purification kits to isolate and purify DNA (New England Biolabs, catalog number: T1110). DNA was eluted with ddH_2O equilibrated to 56 ºC.

To isolate a high concentration of pSC5 (~56 kb) from *E. coli*, alkaline lysis was performed using 27 ml of culture (1.5 ml culture per lysis reaction, 18 reactions in total). The DNA pellets were resuspended with ~25 µl of sddH_2O and combined into a single 1.5 ml tube (~460 µl total). Half of the isolated DNA was then digested using *Sac*I-HF, as described below. Then, a tenth volume of sodium acetate (3M, pH 5.2) and two volumes of ice-cold 100% ethanol were added to the samples. The solutions were inverted to mix and placed into a -80 ºC freezer for an hour before performing centrifugation at 16,000 x G for 10 minutes at 4 ºC. The DNA pellets were then washed twice with 500 µl of ice-cold 70% ethanol before being decanted, dried, and resuspended with 50 µl of sddH_2O. This additional sodium precipitation step was performed to further concentrate the samples and reduce the carryover of contaminants, particularly from the digest reaction.

DNA concentrations were measured by fluorometry using the DeNovix dsDNA Broad Range Assay (FroggaBio, catalog number: DSDNA-BROAD-EVAL) and purity ratios were assessed using a Nanodrop spectrophotometer. For the experiments shown in Fig. 2 to 4, samples were adjusted to be equimolar prior to conducting electroporation.

Restriction digests of episomes

Circular pPtGE31_ ΔPtR was isolated a *P. tricornutum* transformant and recovered in EPI300 *E. coli*. After isolating the episome from *E. coli*, approximately 10 µg was digested using 3 µl of *Eco*RI-HF (New England Biolabs, catalog number: R3101) at 37 ºC for 1 hour. An additional reaction was conducted where 10 µg was digested using 3 µl of *Eco*RI-HF and 3 µl of mung bean nuclease (New England Biolabs, catalog number: M0250) at 37 ºC for 1 hour. Complete digestion was confirmed by agarose gel analysis, then samples were purified using a DNA Cleanup Kit (New England Biolabs, catalog number: T1030).

Circular pSC5 was isolated from EPI300 *E. coli* as described above. Prior to conducting the sodium acetate precipitation step, approximately 40 µg of pSC5 was digested with 18 µl of *Sac*I-HF (New England Biolabs, R3156) at 37 ºC for 1 hour. Complete digestion was confirmed by agarose gel analysis, then the sample was purified by sodium acetate precipitation, as described above.

Assembly of pPtGE31_ShBle

The episome pPtGE31_ShBle was created by yeast assembly of three overlapping fragments. The episome pPtGE31_ ΔPtR was amplified as two fragments using primers that would add 40 bp overlaps for the promoter and terminator region of the ShBle cassette and split the episome in the HIS3 region. The ShBle cassette was amplified from the p0521s plasmid[7] using primers that would add 40 bp overlaps for

the insertion site in pPtGE31_ ΔPtR (all primers are listed in Supplementary Table S6). Yeast assembly was conducted as previously described[27] using ≥ 100 ng of each fragment in place of a bacterial donor.

Electroporation to P. tricornutum

P. tricornutum cells were grown in liquid media or on agar plates for the experiments conducted in Fig. 1 – all successive experiments used cells derived from agar plates. For liquid cultures, 1 ml of cells adjusted to 1 – 2 x 10^8 cells/ml were passaged into 50 ml of L1 media and placed in a growth chamber on a shaker set to ~100 rpm. For agar plate cultures, 250 µl of cells adjusted to 1 x 10^8 cells were spread onto a ½-salt L1 plate with 1% agar (w/v). Liquid and agar plate cultures were grown for 4 days. After 4 days, cells were scraped from the agar plates using 750 µl of L1 and transferred to a 1.5 ml tube. An additional 750 µl of L1 was used to remove any remaining cells from the plate. Then, 10^{-1} to 10^{-2} dilutions of the liquid and agar plate cultures were prepared and used to estimate cell density with a hemocytomer. Liquid and agar plate cultures were adjusted to have a final cell density between 2 – 4 x 10^8 cells/ml; for the liquid cultures, this requires concentrating the cells via centrifugation and resuspension in a smaller volume. Here, the cells are transferred from the flask to a 50 ml conical tube and pelleted by centrifugation at 2000 x G for 10 minutes at 18 ºC. The pellet was resuspended with 375 mM D-sorbitol to a concentration between 2 – 4 x 10^8 cells/ml and transferred to a 1.5 ml tube. Cells derived from agar plates were pelleted by centrifugation at 750 x G for 4 minutes; here, we used a microcentrifuge, which could pellet the cells at a lower centrifugal speed. As before, the cells were resuspended using 375 mM D-sorbitol to a concentration between 2 – 4 x 10^8 cells/ml. The final cell concentration was equalized between the liquid and agar plate derived cultures for every biological replicate.

To spheroplast, 1 mAnson of alcalase (Sigma-Aldrich, catalog number: 126741) was added for every 1 x 10^8 cells. At this stage, the cells will have been previously concentrated to a density between 2 – 4 x 10^8 cells/ml and already resuspended in 375 mM D-sorbitol. Alcalase was diluted 10-times in sddH_2O to reduce the pipetting error associated with small volumes. After the addition of alcalase, cultures were placed on a rocking shaker set to ~15 rpm at room temperature for 20 minutes, during which spheroplasting occurs. Once 20 minutes has passed, the cells were pelleted by centrifugation at 325 x G for 4 minutes. The supernatant is decanted, and the cells are gently resuspended with 1 ml of ice-cold 375 mM D-sorbitol using a P1000 pipette.

The electroporation protocol we used is adapted from Zhang and Hu[6], with adjustments made by Kassaw et al[16] and Pampuch (unpublished). All materials (i.e., cells, DNA, D-sorbitol, electrocuvettes) should be kept on ice for the duration of this protocol. First, the spheroplasted cells were pelleted by centrifugation at 325 x G for 4 minutes. The supernatant is decanted, and the pellet is gently resuspended with 1 ml of ice-cold 375 mM D-sorbitol. This process of centrifugation and resuspension is

repeated another 2 times, such that in total, the cells will have been resuspended with 375 mM D-sorbitol five times (this includes centrifugation steps prior to and post spheroplasting). After the fifth wash, the cells are spun at 750 x G for 3 minutes and a P200 pipette is used to gently remove any remaining supernatant. The cells are gently resuspended with 100 to 200 µl of 375 mM D-sorbitol such that the total volume of resuspended cells will be between ~150 to 250 µl. Into sterile 1.5 ml tubes, 50 µl of cells are aliquoted along with 4 µl (10 µg/µl) of single stranded salmon sperm DNA (ssssDNA, Sigma-Aldrich, catalog number: D7656) and the DNA to be transferred. Of note, the ssssDNA should be heated to 95 ºC for 2 to 5 minutes and then cooled on ice prior to this to ensure it remains single stranded. We used between 1 to 1000 ng of DNA for all transformations. Negative controls consisted of only ssssDNA in the cell mixture.

Cells are gently pipetted up and down 2 times before transferring to an ice-cold sterile electrocuvette (Fisher Scientific, catalog number: FB102), ensuring that no air bubbles have been transferred and the cells lie evenly across the bottom. The electrocuvette is pulsed in an electroporator (BioRad, Gene Pulser Xcell System, catalog number: 1652660) set to the following parameters – voltage: 500 V, capacitance: 50 µF, and resistance: 400 Ω. The time constant varied from 30 to 32.5 milliseconds for the experiments conducted in Figures 1 to 4. Immediately after pulsing, 1 ml of L1 media is gently added to the electrocuvette without disturbing the cells. The electrocuvettes are left to rest at room temperature for 10 minutes before fully resuspending the cells and transferring the mixture to 10 ml of L1 in 50 ml falcon tubes. The lids of the falcon tubes are left loose for air transfer, and the cultures are moved to a growth chamber to recover for 16 to 24 hours.

After recovery, the cultures are pelleted by centrifugation at 2000 x G for 10 minutes at 18 ºC. The pellet is resuspended with 500 µl of L1 media, then 50 µl (one-tenth), 125 µl (a quarter), and/or 250 µl (half) of the cells are spread onto ¼-salt L1 plates supplemented with the appropriate antibiotic(s).

It is worth noting that there is a great degree of variance between electroporation equipment, and this can drastically impact the efficiency of transformation to *P. tricornutum*. If others attempt this protocol, it may be necessary to initially attempt electroporation with several different parameters to determine which settings work best with different set-ups. For our set-up (500V, 50 µF, 400 Ω), an empty electrocuvette generates a time constant of ~35 milliseconds. If the time constant generated by a different machine with the same parameters is lower than 30 milliseconds, we recommend increasing the capacitance (e.g., 75 µF); if it is higher than 40 milliseconds, we recommend decreasing the capacitance (e.g., 25 µF).

Screening P. tricornutum colonies transformed with two fragments

Non-overlapping fragments from pPtGE31_ShBle were electroporated both individually and simultaneously into *P. tricornutum* (Supplementary Fig. S3). Cells that were electroporated with both fragments were plated onto ¼-salt L1 plates supplemented with 100 µg/ml nourseothricin or 100 µg/ml zeocin. Transformants that appeared following electroporation with both fragments were passaged twice – first, onto media supplemented with the same antibiotic as the initial transformation, and then onto media supplemented with the alternative antibiotic (Supplementary Table S5). Twenty transformants, ten derived from each initial transformation plate, that demonstrated resistance to both nourseothricin and zeocin were passaged for a third time onto double-selection media (¼-salt L1 supplemented with 50 µg/ml nourseothricin and zeocin). DNA isolation was performed using cells from this third passage, followed by PCR screening.

Recovery of P. tricornutum episomes in E. coli

Episomes were isolated from *P. tricornutum* transformants as described above. For each electroporation reaction, 2 to 4 µl of *P. tricornutum* DNA was combined with 25 to 50 µl of homemade electrocompetent EPI300 cells (derived from LGC Biosearch Technologies, catalog number: EC02T110) in a sterile 1.5 ml tube. The mixture was gently pipetted twice and then transferred to a sterile 2 mm electrocuvette, ensuring no air bubbles were transferred. The electrocuvette is pulsed in an electroporator set to the following parameters – voltage: 2.5 kV, capacitance: 25 µF, and resistance: 200 Ω. This generated a time constant of 4.9 to 5.2 milliseconds. Electroporated cells were resuspended by pipetting 1000 µl of SOC media up-and-down in the electrocuvette, and then transferred to sterile 1.5 ml tubes to recover at 37 °C and 225 rpm for approximately 1 hour. After recovery, half of the reaction volume (~400 µl) was spread across a 1.5% agar (w/v) LB plate supplemented with 15 µg/ml chloramphenicol (pPtGE31_ ΔPtR and pPtGE31_ShBle recovery) or 40 µg/ml gentamycin (pSC5 recovery), which were then transferred to a 37 °C incubator. Transformants appeared across the plates within 24 hours, with there being a range from 4 to ~100 colonies per transformation.

<u>For recovery of pPtGE31_ ΔPtR and pPtGE31_ ShBle</u>: single *E. coli* colonies were picked and inoculated into 5 ml of LB media supplemented with 15 µg/ml chloramphenicol. Cultures were grown overnight at 37 ºC and 225 rpm. The following day, 500 µl of culture was inoculated into 4.5 ml of LB media supplemented with 15 µg/ml chloramphenicol and 100 µg/ml L-arabinose. These episomes contain a pCC1BAC backbone, which can be induced to high-copy number replication in EPI300 through by adding L-arabinose to the media. Induced cultures were grown for at least five hours prior to episome isolation, with 1.5 ml of culture being used for DNA isolation, as described above.

<u>For recovery of the conjugative plasmid pSC5</u>: DNA isolated from eight *P. tricornutum* pSC5 transformants were electroporated into EPI300 cells. Four colonies per

transformation were inoculated into 5 ml of LB supplemented with 40 µg/ml gentamycin. Additionally, EPI300 strains carrying pSC5 (positive control) or pSAP (recipient cell line) were inoculated from glycerol stocks into 5 ml of LB supplemented with 40 µg/ml gentamycin (pSC5) or 15 µg/ml chloramphenicol (pSAP). The cultures were placed at 37 ºC and 225 rpm. After the cultures reached saturation (~18 hours), 125 µl of the pooled *E. coli* transformants and pSC5 cultures were inoculated into 5 ml of LB supplemented with 40 µg/ml gentamycin. For the pSAP culture, 5 ml of culture was diluted into 45 ml of LB supplemented with 15 µg/ml chloramphenicol. The cultures were grown at 37 ºC and 225 rpm for 2 hours. Then, the cultures were pelleted by centrifugation at 3000 x G for 12 minutes at 10 ºC, decanting the supernatant post centrifugation. The pooled transformants and pSC5 cultures were each resuspended with 100 µl of 10% glycerol, whereas the 50 ml pSAP culture was resuspended with 1000 µl of glycerol. Strains were kept on ice during these steps to cease bacterial growth.

For conjugation, 100 µl of the donor cells (transformant pools [x8] and pSC5) were mixed with 100 µl of the recipient cells (pSAP). The cells were mixed by pipetting up and down 4 times before transferring the whole mixture to a 2% agar (w/v) LB plate. Additionally, 100 µl of the recipient was plated by itself to serve as a negative control. Conjugation plates were incubated at 37 ºC for 1 hour. Then, plates were scraped using 2 ml of sddH_2O. The scraped cells were vortexed for 5 seconds to ensure any clumps were dispersed. Transconjugants were spot plated onto 1.5% agar (w/v) LB plates supplemented with 40 µg/ml gentamycin and 15 µg/ml chloramphenicol with dilutions ranging from 10^0 to 10^{-8}.

Sequencing and alignment

Amplicons generated by amplifying the episome termini region in *P. tricornutum* transformants were sent for Sanger sequencing (London Regional Genomics Centre). Episomes isolated from *E. coli* were sent for Oxford Nanopore whole-plasmid sequencing (Plasmidsaurus Inc). The resulting sequences were manually aligned to the respective template episomes. Regions that could not be manually aligned were queried using a local alignment algorithm (EMBOSS water tool)[28] to search for homology in the episome. Sequences that could not be aligned manually or algorithmically were queried using BLASTn (NCBI)[29].

Analyses of P. tricornutum transformation efficiency

The total number of colonies per transformation reaction were estimated by manually counting the number of CFUs per plate and then multiplying this value by 2 or 10, depending on whether half or a tenth of the total reaction was plated, respectively. In instances where the transformation plate was too dense to reliably count by eye, five 1 cm^2 quadrants were sampled from the plate and counted using a dissecting

microscope with 10x magnification. Here, the total number of CFUs was estimated by averaging the number of colonies per quadrant and multiplying this value (i.e., colonies per cm^2) by the total surface area of the petri dish.

Transformation efficiencies were estimated by dividing the total number of CFUs by the number of cells per reaction. When graphically comparing transformation reactions, all calculations and plotting were done in R; otherwise, we used an Excel spreadsheet keep track of data and tabulate transformation efficiencies. The values for the standard error of the mean were calculated by dividing the standard deviation for a series of experiments by the square root of the number of biological replicates.

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AUTHOR CONTRIBUTIONS

MP and GT established the parameters necessary for conducting electroporation in the Karas lab and performed experiments 1 through 79 in Supplementary Table 1. EJW established the spheroplasting technique and performed all other experiments detailed in the manuscript. EJW wrote the manuscript and created all the figures in Adobe Illustrator; MP and BJK edited the manuscript and provided crucial insight. BJK supervised all experimentation done by EJW, MP, and GT, and assisted with experimental design, data analysis, troubleshooting, and more.

COMPETING INTEREST DECLARATION

The authors declare no competing interests.

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