The genetic intractability of *Symbiodinium microadriaticum* to standard algal transformation methods

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Abstract

Modern transformation and genome editing techniques have shown great success across a broad variety of organisms. However, no study of successfully applied genome editing has been reported in a dinoflagellate despite the first genetic transformation of *Symbiodinium* being published about 20 years ago. Using an array of different available transformation techniques, we attempted to transform *Symbiodinium microadriaticum* (CCMP2467), a dinoflagellate symbiont of reef-building corals, in order to perform CRISPR-Ca9 mediated genome editing. Plasmid vectors containing the chloramphenicol resistance gene under the control of the CaMV p35S promoter as well as several putative endogenous promoters were used to test a variety of transformation techniques including biolistics, electroporation, silica whiskers and glass bead agitation. We report that we have been unable to confer chloramphenicol resistance to our specific *Symbiodinium* strain. These results are intended to provide other researchers with an overview of previously attempted techniques and sequences in order to support efficient planning of future experiments in this important field.

Introduction

Efforts to better understand the molecular mechanisms which govern the symbiosis between marine Cnidarians and their dinoflagellate symbionts have been hampered by the lack of genetically tractable model organisms. This is especially true for the symbiotic relationship between corals and dinoflagellates from the genus *Symbiodinium*. This interaction forms the bedrock of the coral ecosystem (1, 2) and yet is highly sensitive to relatively small changes in environmental conditions (3). Abnormally high oceans temperatures have been identified as one of the key factors that can precipitate the breakdown of the *Symbiodinium*-coral symbiosis, which can lead to wide-spread, regional and even global coral bleaching events (4). The predicted increase in oceans temperatures due to anthropogenic climate change is expected to accelerate this crisis (5).

One of the prevailing theories on how coral reefs would be able to withstand rising ocean temperatures rests on the assumption that there are certain thermo-tolerant *Symbiodinium* strains which are able to form a more robust relationship with their host (6). While the effectiveness of thermo-tolerant *Symbiodinium* strains in maintaining algal-coral symbiosis has been shown to be significant, the genetic basis of this robustness remains unknown. Without genetic tools and the absence of any viable method to carry out traditional genetic studies such as inbreeding and cross-breeding, isolating and confirming the identity of thermo-tolerance genes will be difficult.

Previous studies described methods for transformation of free-living *Symbiodinium* cells. The first, published in 1997 by Ten Lohuis and Miller describes the transformation of *Symbiodinium* CS-153 using silicon carbide whiskers. In the Ten Lohuis paper, it was reported that the Caulimoflower Mosaic Virus p35S, nos and p1'2' promoters were able to drive the expression of reporter genes (GUS) and selectable markers (hygromycin and geneticin resistance genes) in *Symbiodinium*. The second, published more recently in 2015, used agrobacterium-mediated glass bead agitation to transform *Symbiodinium kawagutii*, *Symbiodinium* sp. Mf11.5b.1 and *Symbiodinium microadriaticum* MAC-CassKB8. In this publication, the authors used the *nos* promoter to drive the expression of the bar gene which confers resistance to the herbicide Basta. However, the authors of the paper note

that their transformed cells lost their chlorophyll and were unable to reproduce under herbicide selection.

We have used previously published (Ref Lohuis) transformation protocols for *Symbiodinium* as well as different standard protocols for alga based on electroporation and biolistics to transform *S. microadriaticum* CCMP2467. Plasmid constructs utilizing the Cauliflower Mosaic Virus p35S promoter as well as putative endogenous *Symbiodinium* promoter and terminator regions identified from the S. *microadriaticum* genome (7) were used to drive the chloramphenicol resistance gene. However, we were unable to obtain resistant strains under chloramphenicol selection. We report our findings with the intention that future attempts at *Symbiodinium* transformation will be better informed about protocols and conditions that have already been tested.

Materials and Methods

Symbiodinium cell culture conditions

Cultures of the dinoflagellate *Symbiodinium microadriaticum* (strain CCMP2467) were obtained from the Bigelow National Center for Marine Algae and Microbiota (NCMA). This strain was originally isolated from a scleractinian coral, *Stylophora pistillata*, in the Gulf of Aqaba (8, 9). Stock cultures were grown in Percival incubators under a 12:12 day:night regiment in f/2 media in Nunc cell culture-treated TripleFlasks (132913) (ThermoFisher Scientific, Waltham, MA). Growth conditions were set at a Photosynthetic Photon Flux Density of 80 umol photons m^{-2} s^{-1}, growth temperature of 26 °C and growth media salinity of 40 ppt (i.e. the salinity of seawater from the Red Sea). These growth conditions will be referred to henceforth as standard culture conditions.

Transformation methods

Silicon carbide whiskers agitation transformation

This protocol was modified from Ten Lohuis and Miller (1998) (10). Approximately 5×10^7 cells were harvested by centrifugation at 3000*g* for 5 minutes, washed with 5 ml of f/2 media, repelleted, and then resuspended in 500 µl of f/2 media. A transformation mixture containing 40 µl of 50 µg/ml Silar silicon carbide whiskers (997002-5g) (Haydale Technologies Incorporated, Greer, South Carolina, USA) was sequentially mixed with 20 to 40 µg of plasmid (circular or linear), 160 µl of PEG8000 (20% wv; filter sterilized) and then topped up with f/2 media to a final volume of 250 µl. The 500 µl resuspended cells were then added to the transformation mixture and vortexed over a period of 2 minutes, pausing for 5 seconds every 10 seconds. 2 ml of f/2 with 100 µg/ml of carbenecillin was added to the transformation mixture. The mixture was then incubated in the dark at 27 °C for 1 to 2 days. The agitated *Symbiodinium* were then grown under selection in either liquid culture (1 ml of transformant culture in 150 ml of liquid f/2 media) or on agar plates (150 µl of transformant culture per agar plate).

Biolistics

Biolistics protocol was modified from instructions given in the Biorad PDS-1000/He Biolistic Particle Delivery System manual. Approximately 5×10^7 Symbiodinium cells were harvested by centrifugation at 3000*g* for 5 minutes and subsequently washed with 5 ml of filtered, sterilized f/2 before being plated onto an f/2 agar plate approximately 1 hour before transformation. Tungsten or gold microcarriers suspended in 50% glycerol (30 mg/ml) were vortexed for 5 minutes before 50 µl of the mix was aliquoted into a 1.5 ml Eppendorf tube. 5 µg of plasmid DNA was added to the microcarrier mix and immediately vortexed for 10 seconds. This was followed by the addition of 50 µl of 2.5 M $CaCl_2$, vortexing for 10 seconds, and the addition 20 µl 0.1 M spermidine. The mixture was then mixed via vortexing for 2 minutes before being allowed to settle for 1 minute. The microcarriers were then pelleted by pulse centrifugation and the liquid supernatant was discarded. The pellet was gently washed with 140 µl of 70% ethanol before the mix was pulse centrifuged and the supernatant was removed. This washing step was repeated with 140 µl of 100% ethanol. The pellet was then re-suspended with 10 or 70 µl of 100% ethanol depending on the number of macrocarriers being used. 10 µl of the mix was used to dip dry a layer of microcarriers onto a Biorad biolistics macrocarrier (#1652335) (Biorad, Hercules, California, US) and this step was repeated seven times when using 7 macrocarriers. The Biorad macrocarrier was then loaded into a macrocarrier holder and fired at an agar plate of *Symbiodinium* cells as according to the manufacturer's manual for the Biorad PDS-1000/He Biolistic Particle Delivery System. Chamber pressure, rupture disk pressure and distance of agar plate from the macrocarrier holder were adjusted based on the experiments carried out as described in the Results section.

After the biolistics firing, *Symbiodinium* cells were transferred from the agar plate into 5 ml of f/2 media and incubated under standard culture conditions for one day. The transformed *Symbiodinium* were then grown under selection in liquid culture (2 ml of transformant culture in 150 ml of liquid f/2 media) and/or on agar plates (150 µl of transformant culture per agar plate).

Electroporation

The electroporation protocol used in this publication was modified from published protocols used in *Phaeodactylum tricornutum* and *Nannochloropsis* sp. (11, 12). Approximately 1×10^8 cells were harvested by centrifugation at 3000*g* for 5 minutes. The cells were subsequently washed with 5 ml of f/2 and repelleted, before being washed with 1 ml of 375 mM sorbitol, repelleted, and finally resuspended in a 100 µl of 375 mM sorbitol. The suspension was then mixed with 2-4 µg of plasmid DNA and 40 µg of denatured salmon sperm DNA. The mixture was then incubated on ice for 10 minutes before being transferred into a 2-mm electroporation cuvette. Electroporation was performed using a Bio-Rad Gene Pulser Xcell Electroporation system. The parameters of the system (exponential decay vs multiple pulse, field strength, capacitance, shunt resistance) were adjusted according the experiments carried out as described in the Results section.

After electroporation, the cells were immediately transferred to a 15 ml Falcon tube containing 10 ml of f/2 media with 100 µg/ml carbenicillin and left under standard cultures conditions for one day. After this, the cells were then collected by centrifugation at 1500 *g* for 10 minutes and resuspended in 3 ml of f/2 media. The electroporated *Symbiodinium* were then grown under selection in liquid culture (1 ml of transformant culture in 150 ml of liquid f/2 media) and/or on agar plates (150 µl of transformant culture per agar plate).

Glass bead agitation transformation, with cell wall digest step

The glass bead protocol was modified from Kindle (1990) (13) and the cellulase cell wall digest was modified from Levin *et al.* 2017 (14). Approximately 2×10^6 cells were harvested by centrifugation at 3000*g* for 5 minutes, washed with 5 ml of f/2 media, repelleted and then resuspended in 1 ml of digestion solution (0.5 M D-sorbitol in filtered autoclaved seawater). Either 0.3 kilounits (KU) of cellulase from *Trichoderma* sp. (C1794, Sigma-Aldrich) or 10 mg of Snailase (S0100, Beijing Biodee Biotechnology Co., Ltd) was added to the digest mix. The mixture was then incubated in the dark at 30 °C for 1 day on a tube rotator.

The digest mixture was then centrifuged at 800*g* for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1 ml of protoplast wash solution (0.5 M D-sorbitol, 0.5 M sucrose, 25 mM $CaCl_2$, and 100 mg/ml kanamycin in filtered autoclaved seawater) before being incubated at 25 °C for 3 hours in the dark on a tube rotator. The cells were centrifuged at 800*g* for 5 minutes before being washed with 1 ml of f/2 media, repelleted, and resuspended in 300 µl of f/2 media.

100 µl of filter sterilized 20% w/v PEG 6000 (81260, Sigma) was added to the 300 µl of resuspended cells followed by the addition of 50 µg of salmon sperm carrier DNA (ref) and 2 µg of plasmid DNA (in various combinations of circular and linearized plasmids, as described in the results section). 300 µg of autoclave-sterilized glass beads 0.5 mm in diameter (BioSpec) were added to the transformation mixture. The mixture was vortexed for 30 seconds after which the beads were allowed to settle. The agitated *Symbiodinium* were then resuspended in 2 ml of fresh f/2 with 100 µg/ml of carbenicillin. After this, the transformant cultures were grown under selection in liquid media (1 ml of transformant culture in 150 ml of liquid f/2 media) and on agar plates plates (150 µl of transformant culture per agar plate).

FuGENE HD transfection

Approximately 5×10^6 *Symbiodinium* cells were harvested by centrifugation at 3000*g* for 5 minutes, washed with 5ml of f/2 media, repelleted and resuspended in 150 µl of f/2 media. A 30 µl FuGENE transfection/DNA mixture was made using f/2 media with different mixtures of plasmid DNA and FuGENE HD transfection reagent as according to Supplementary Table T5D. 1 to 30 µl of this mix was then added to the 150 µl cell suspension and incubated at standard growth conditions for 24 hours. The cells were then grown under selection in liquid culture (100 µl of transformant culture per 150 ml of liquid f/2 media).

f/2 agar plate selection

1.5 % agar made using filtered, autoclaved seawater and enriched with f/2 media was used to make agar plates for transformation selection. 100 µg/ml chloramphenicol was used as the selection antibiotic depending on the construct being tested. Control plates with no selection antibiotic was also made to confirm that the transformation method was mild enough that some cells survived the treatment. The samples were inoculated onto the agar plate using 3 ml of top agar (0.5 % agar in seawater). This was done due to the tendency of *Symbiodinium* cells to clump, which prevents the cells from being easily evenly spread across agar surfaces. The plates were then grown under standard culture conditions for up to four months.

Liquid f/2 selection

Transformant cultures were grown in 225 cm^2 EasYFlask Nunc bottles (159934, Thermo Scientific, Waltham, Massachusetts, USA) filled with 150 ml of filtered, autoclaved seawater enriched with f/2 media. The selection antibiotic used was 100 µg/ml chloramphenicol and the cultures were kept under standard growth conditions.

Results

Antibiotic susceptibility test

Previous studies (1998) used notably high concentrations of hygromycin and geneticin (G418), 3 mg/ml, in order to select for resistant *Symbiodinium* transformants, which required about three months as untransformed cells were able to survive up to eight weeks of exposure.

In order to select a more effective selection antibiotic, we used liquid cultures to test the effectiveness of several antibiotics to determine a more potent and less expensive alternative to hygromycin and geneticin. We determined that 100 µg/ml of chloramphenicol was just as effective as 3 mg/ml of hygromycin in reducing the number of observed *Symbiodinium* cells in culture (Figure 1). In addition, the cost of chloramphenicol is significantly lower than hygromycin weight-for-weight across a range of suppliers (Supplementary Table 1). Therefore, we decided to use chloramphenicol instead of hygromycin to select for our transformants.

Test for chloramphenicol resistance gene function

We first tested the functionality of the chloramphenicol resistance gene using *Saccharomyces cerevisiae* expression vectors in *S. cerevisiae*. Under galactose induction, yeast cultures were grown with and without chloramphenicol selection (4 mg/ml)(15) to show that the presence of the ChloR gene under the control of the GAL promoter was necessary and sufficient to confer increase chloramphenicol resistance to yeast cells. The minimal growth media used for this experiment was supplemented with 3% glycerol and 0.5% galactose in order to force yeast cells to grow anaerobically while providing a low level of galactose to induce GAL promoter expression. The results show that yeast cells transformed with pYESChloR constructs which contains the ChloR gene under the control of the GAL promoter were able to grow at comparable rates with cultures that did not contain chloramphenicol (Figure 2). Parental type (ΔMEP) *S. cerevisiae* cells and cells transformed with pYESeGFP constructs on the other hand had reduced growth rates in the presence of chloramphenicol.

Promoter region identification

In order to identify suitable promoter regions we selected a set of 5 highly expressed genes including (β-tubulin A, β -tubulin B, Hsp90, Actin and *PsbJ* from the *S. microadriaticum* genome. For the nuclear constructs based on the β-tubulin A, β -tubulin B and Hsp90 promoters, we took the 3 kb region upstream of the predicted start codon as the putative promoter region (Supplementary Data D9 and D10). We also used a 400 bp 3' region originating downstream of the endogenous Hsp90 stop codon as the terminator region for the pHsp90-ChloR-Hsp90T construct. . To identify the right start codon for the Actin and *PsbJ* genes we performed 5' RACE on transcripts to sequence their 5' UTRs. For the Actin gene, the 5' RACE results confirmed the presence of a splice leader 55 bp upstream of the first ATG site (Figure 3). This particular splice leader sequence is notable for being the most common splice leader sequence in the *Symbiodinium kawagutii* transcriptome, being found in 6235 out of 6501 full-length *S. kawagutii* cDNAs containing a complete splice leader sequence (16). Using the coding sequence, we did a BLAST search on the *S. microadriaticum* genome (7) and identified gene model Smic17360 as the best hit sequence for our 5' RACE result. We then amplified an approximately 2 kb region upstream from the putative start ATG to serve as the promoter element for the pAct-ChloR-ActT construct. The 3' terminator sequence used for this construct was the 500 bp region downstream of the stop codon of Smic17360.

Within the 2 kb promoter region upstream of Smic17360 (Actin), we identified all promoter elements that have been noted as important for proper splice leader splicing (Figure 4A) (17). This includes a TTTT-box motif 34 bp upstream of a potential transcriptional start site (YYANWYY) and a branch point (YTNAY) 32 bp upstream of the splice acceptor (AG) (Figure 4B).

We carried out a similar 5' RACE validation experiment to define the *PsbJ* promoter (*pPsbJ*) region (Supplementary Figure 1). In most photosynthetic organisms, the *psbJ* gene is located in the chloroplasts genome as part of the *psbEFLJ* operon. However, in the *S. microadriaticum* genome the *PsbJ* gene is nuclear-encoded, which lends support to previous reports that *psbJ* is not encoded on any of the *Symbiodinium* chloroplasts minicircles. In addition, *psbE* in the *Symbiodinium* chloroplast

genome is not found in an operon but rather was found to not be co-localized with any other gene. For these reasons, the *pPsbJ-ChloR* construct promoter region was designed to contain additional N-terminal peptides which are absent in all other, chloroplast-encoded orthologues of *PsbJ* and which could be *Symbiodinium* chloroplasts localization peptides (Supplementary Figure 2). Note that because the *Symbiodinium* PsbJ is encoded in the nucleus, we are using the gene name format for higher plant nuclear genes (capitalized first letter, *PsbJ*) rather than the naming format for plastid genes (lower case first letter, *psbJ*).

Transformation construct design

Plasmids were constructed to carry the chloramphenicol (*ChloR*) resistance gene under the control of a series of different gene promoters and terminators (Table 1). We use the term *ChloR* in our paper to describe the chloramphenicol acetyltransferase (*CAT*) gene which confers resistance to chloramphenicol.

Chloramphenicol constructs, derived from the pCR2.1 vector backbone, were designed for nuclear *Symbiodinium* transformation using endogenous promoters, and in some cases, endogenous terminator. In addition to the endogenous promoter and terminator regions previously described, the p35S promoter and Nos terminator were also used to construct the p35S-ChloR-NosT construct (Figure 5), as it has been reported previously that these expression elements were sufficient to drive the expression of the hygromycin resistance gene in *Symbiodinium*.

Transformation details

A brief summary the various transformation experiments carried out can be seen in Table 2, and the details of each transformation can be found in Supplementary Table . 38 sets of transformation experiments were carried out for a total of 190 transformation samples, including control samples (i.e. no plasmid control). Various transformation conditions for each transformation protocol were tested, and all transformants were kept under observation for at least 3 months. Any cultures which showed signs of growth were sub-cultured into fresh f/2 liquid or agar with the appropriate selection antibiotic/herbicide. Although we did observe that some of these cultures began to grow after

several months in liquid culture, none of these “transformant lines” were able to grow after sub-culturing in fresh selection media (data not shown).

None of the transformant cultures tested on selection agar plates survived more than four weeks. We also noticed that cells on the controls plates without selection did not survive for more than six weeks after many of our transformations treatments. This is unlike the situation in our liquid media cultures, where some control cultures could be kept viable for around three months. Due to this, most of our screening results were only carried out using liquid media in order to use the control liquid culture to provide an acceptable end-point for experimental observation (I.e. transformations were considered to have failed when the no-antibiotic/no-herbicide control cultures became visibly decoloured due to senescence).

Discussion

Our attempts to stably transform *S. microadriaticum* CCMP2467 have not been successful despite the use of a wide variety of constructs and standard transformation methods known to work successfully in the transformation of other microalgae. We have tested both transgenic (p35S) and endogenous promoters (pAct, pBTubA, pBTubB and pHsp90) to drive ChloR expression in the cytoplasms as well as endogenous promoters of proteins targeted to the chloroplasts (pPsbJA and B).

In term of transformation methods, we have tried to transform *Symbiodinium* using silicon carbide whisker agitation, biolistics (particle bombardment), electroporation and glass bead agitation. In addition, we used FuGENE transfection media to see if methods more commonly used to transfect animal cells could work. We did not test agrobacterium-mediated transformation as a publication by Ortiz-Matamoros et al. (2015) clearly stated that this method, as tested by the authors, was only able to create transient, rather than stable, transformants which were unable to replicate and maintain normal pigmentation under Basta herbicide selection (18).

All in all, we did not observe any successful transformants in either liquid culture or agar plates.

Conclusion

Our results illustrate the resistance of *Symbiodinium* to well-established algae transformation methods. Since the first reported publication by Ten Lohuis and Miller (1998), to our knowledge, the only other primary paper concerning *Symbiodinium* transformations is Ortiz-Matamoros *et al.* (2015), which reported transient expression of GFP in *Symbiodinium* using *Agrobacterium tumefaciens*-assisted glass bead transformations. However, this method was associated with loss of photosynthetic pigments and inability to replicate under Basta herbicide selection. Taken together, these results strongly suggests that this particular algal group may require novel transformation methods and techniques for successful editing of the genome. We publish these results in the hopes that other researcher who wish to work on developing transformation techniques for *S. microadriticum* CCMP2467 in particular, and for *Symbiodinium* in general, be aware of the techniques and conditions reported here that we have worked on and that further effort should be focused on other, untested methods and conditions.

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Figure 1: Growth of *Symbiodinium* in f/2 liquid media under antibiotic selection. The error bars indicate 95% confidence interval. Results were from three biological replicates, each replicate measured four times.

Figure 2: *S. cerevisiae* growth under chloramphenicol selection. Asterisks indicate significantly different means ($p<0.05$) using Student's t-test with unequal variance. T-test was carried out with a significance value of 0.05. Error bars indicate standard error of the mean.

Figure 3: 5' RACE of Actin transcript.

Figure 4: Unique promoter architecture in Symbiodinium. (A) A schematic view of the proposed promoter (TTTT-box) relative to the putative transcription start site, splice branch point and acceptor site, upstream of the coding region (CDS) in the genomic sequence of *S. kawagutii* genes. For comparison, premature mRNA and mature mRNA are also shown. Figure was adapted from Lin et al. (2015). (B) Sequence of *S. microadriaticum pAct* promoter 175 bp upstream of the putative start codon, with promoter elements as described in (A).

Figure 5: Vector map for plasmid p35S-ChloR-NosT, constructed using the pCR2.1 vector backbone.

Table 1: List of plasmid constructs.

Table 2: Summary list of the number of experiments carried out grouped by transformation method. Details on each transformation can be found in Supplementary Table T5.

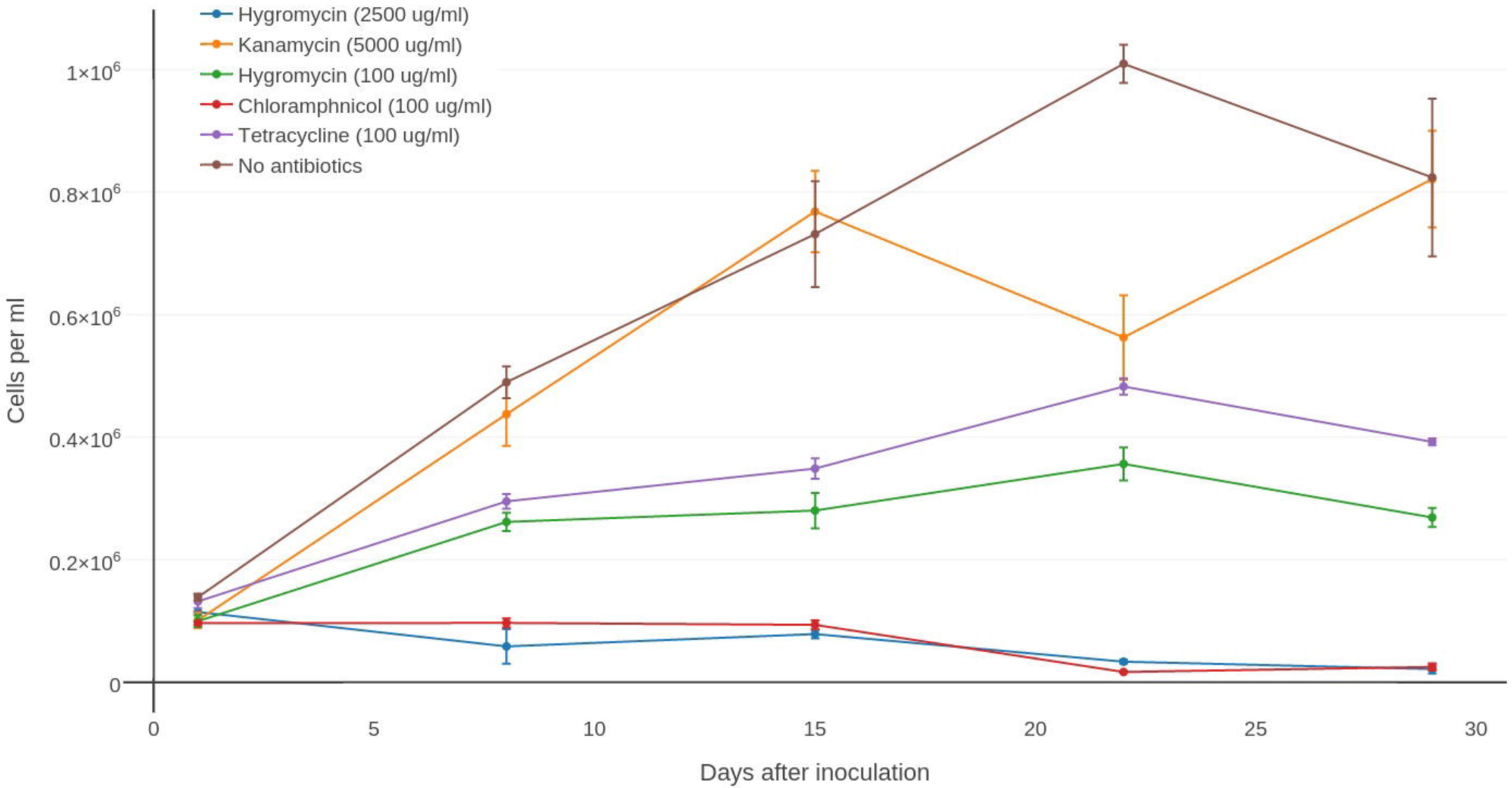
Supporting information

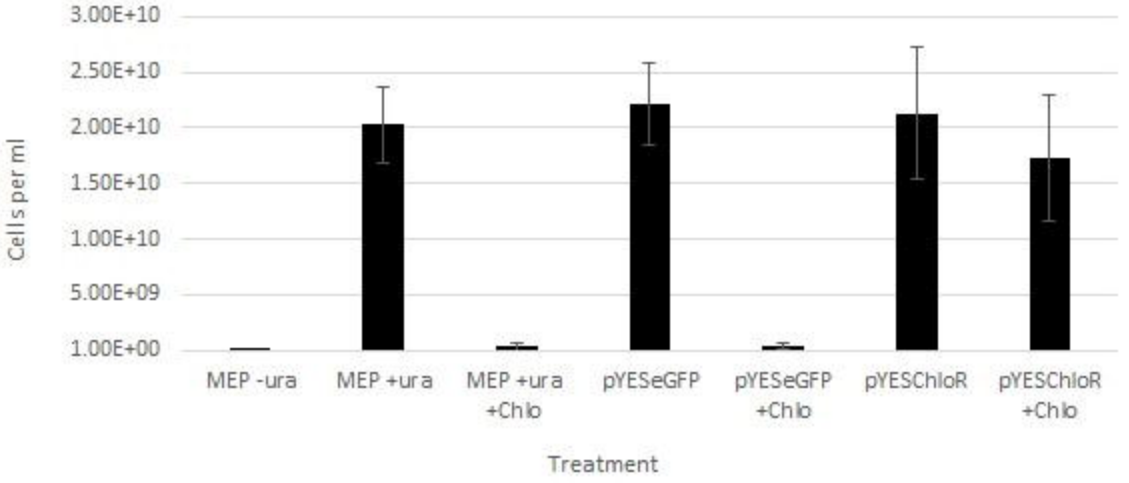
Supplementary Figure 1: Verification of *PsbJ* 5' UTR. (A) Genomic sequence of *PsbJ* gene cluster. Purple underlined sequence indicates sequence that was verified via 5' RACE. (B) 5' RACE of *PsbJ* transcript.

Supplementary Figure 2: Alignment of putative *PsbJ* coding sequences against other *psbJ* orthologues. The *Symbiodinium* putative *PsbJ* gene possesses an alternate in-frame start codon upstream of the start codon found in other *psbJ* homologues, and the N-teminal peptides could be involved in plastidic sub-cellular compartmentalization signaling. The NCBI reference sequence ID are as follows: *Phaeodactylum tricornutum* YP_874372.1, *Arabidopsis thaliana* NC_000932.1, *Arabidopsis lyrata* NC_034365.1, *Nicotiana tabacum* NC_001879.2.

Supplementary Table 1: Prices of antibiotics chloramphenicol, hygromycin and geneticin from various suppliers as of April the 17th, 2017.

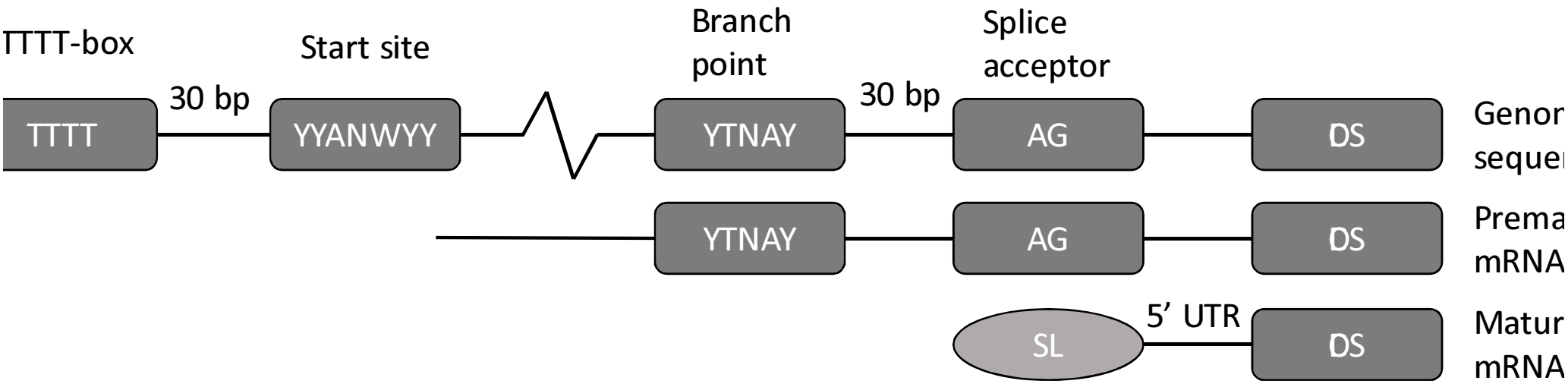
Supplementary Table 2: Detailed list of transformations.


Hygromycin (2500 ug/ml)
Kanamycin (5000 ug/ml)
Hygromycin (100 ug/ml)
Chloramphnicol (100 ug/ml)
Tetracycline (100 ug/ml)
No antibiotics
Cells per ml
1×10^6
0.8×10^6
0.6×10^6
0.4×10^6
0.2×10^6
0
0
5
10
15
20
25
30
Days after inoculation


3.00E+10
2.50E+10
2.00E+10
1.50E+10
1.00E+10
5.00E+09
1.00E+00
Cells per ml
MEP -ura
MEP +ura
MEP +ura +Chlo
pYESeGFP
pYESeGFP +Chlo
pYESChloR
pYESChloR +Chlo
Treatment

GGACACTGACATGGACTGAAGGAGTAGAAA**TCCGTAGCCATTTTGGCTCAAG**TTAG
CCATGTCGGACGAGGTTGTCGCTCTCGTGGTGGACAATGGCAGTGGCATGTGCAAG
GCCGGCTTCTCCGGTGATGACGCTCCGCGAACGGTTTTTCCCTCCCTGATCGGGCG
ACCGAAGCACCGGAGCATTGTGGTGGGGATGGATCAGAGAGACCACTACGTCGGAG
ACGAAGCACAAGCCAAGCGCGGTGTCTTGGCTCTCAAGTACCCTATCGAGCACGGC
ATCGTGACGAACTGGGACGACATGGAGCGGATCTGGCACCACACTTTCTACAA

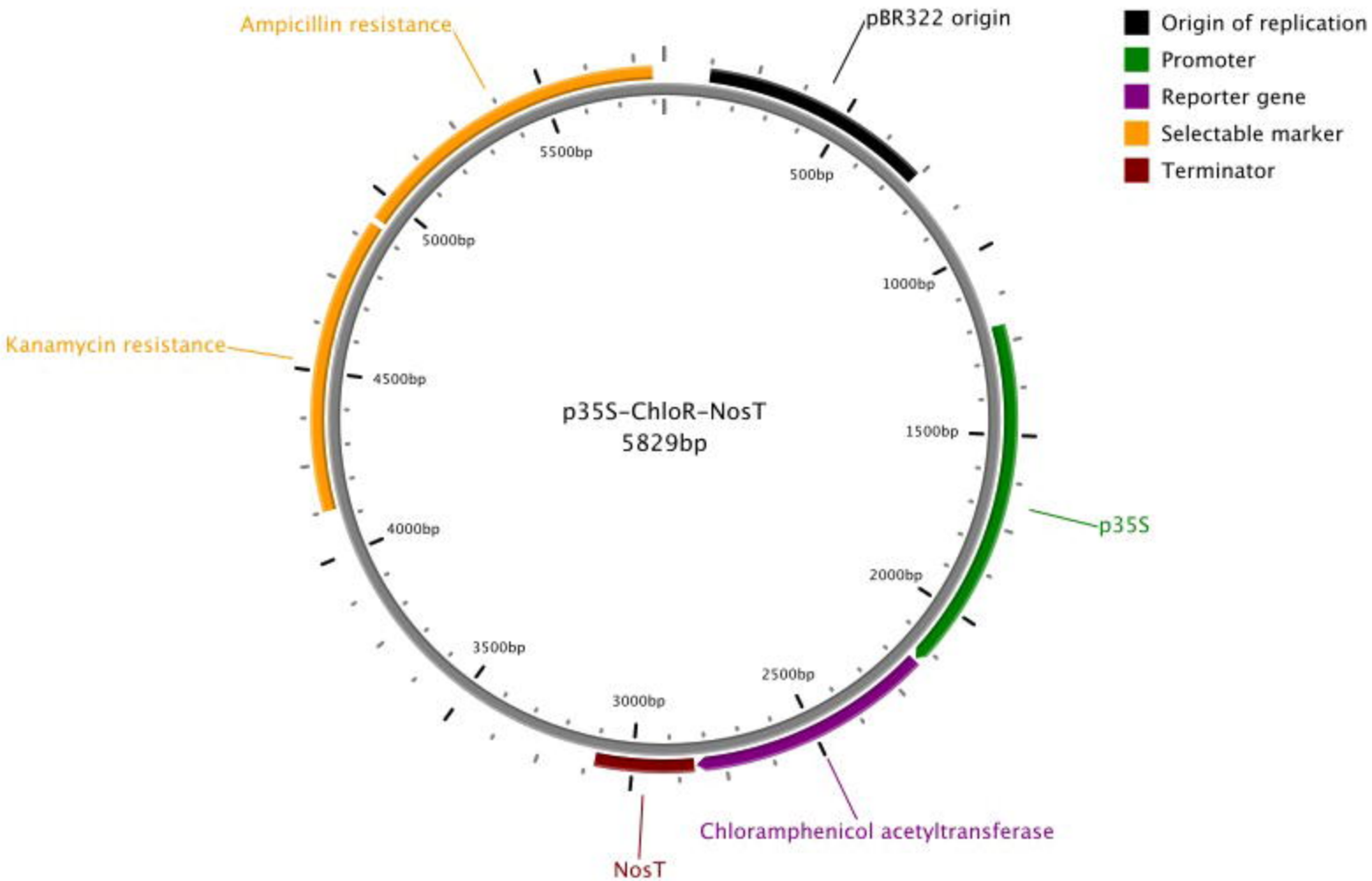
5’ RACE forward primer
5’ UTR
Splice leader
Putative Actin coding sequence
Degenerate Actin reverse primer



TTTT-box Start site

GGATTTTGGGATCGGGTTTAGGGACTGGGGCCCTGAACACCTTAATCCTGCTAGGGTTT
TTTCAGCCCTGTTACAGGGCTTAGAAGGGTTCTTTAAGTTCGGAAGCGGCCAGCATTTC
GGCTTCTCAGATTGACCTCCCGCAGCCTCTTTCCACGAAGTGTCTCGGAGTTAGCCATG Start codon

Branch point Splice acceptor


Ampicillin resistance
pBR322 origin
Origin of replication
Promoter
Reporter gene
Selectable marker
Terminator
5500bp
500bp
5000bp
1000bp
Kanamycin resistance
4500bp
p35S-ChloR-NosT
5829bp
1500bp
p35S
4000bp
2000bp
3500bp
2500bp
3000bp
Chloramphenicol acetyltransferase
NosT

Construct name	Plasmid backbone	Promoter origin	Resistance gene	Terminator origin
p35S-ChloR-NosT	pCR2.1	Cauliflower mosaic virus 35S	Chloramphenicol acetyltransferase	Agrobacterium Nopaline synthase
pAct-ChloR-ActT	pCR2.1	Symbiodinium SmicGene17360	Chloramphenicol acetyltransferase	Symbiodinium SmicGene17360
pBTubA-ChloR	pCR2.1	Symbiodinium SmicGene25527	Chloramphenicol acetyltransferase	(no terminator)
pBTubB-ChloR	pCR2.1	Symbiodinium SmicGene4192	Chloramphenicol acetyltransferase	(no terminator)
pHsp90-ChloR-Hsp90T	pCR2.1	Symbiodinium SmicGene33536	Chloramphenicol acetyltransferase	Symbiodinium SmicGene33536
pPsbJ-ChloR	pCR2.1	Symbiodinium SmicGene31399	Chloramphenicol acetyltransferase	(no terminator)

Transformation method	Number of experiments	Number of transformants
Electroporation	13	53
Biolistics	8	53
Silicon carbide whiskers agitation	5	24
FuGENE transfection	2	24
Glass beads agitation	2	6