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Engineering Strategies to Decode and Enhance the Genomes of Coral Symbionts

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Elevated sea surface temperatures from a severe and prolonged El Niño event (2014–2016) fueled by climate change have resulted in mass coral bleaching (loss of dinoflagellate photosymbionts, *Symbiodinium* spp., from coral tissues) and subsequent coral mortality, devastating reefs worldwide. Genetic variation within and between *Symbiodinium* species strongly influences the bleaching tolerance of corals, thus recent papers have called for genetic engineering of *Symbiodinium* to elucidate the genetic basis of bleaching-relevant *Symbiodinium* traits. However, while *Symbiodinium* has been intensively studied for over 50 years, genetic transformation of *Symbiodinium* has seen little success likely due to the large evolutionary divergence between *Symbiodinium* and other model eukaryotes rendering standard transformation systems incompatible. Here, we integrate the growing wealth of *Symbiodinium* next-generation sequencing data to design tailored genetic engineering strategies. Specifically, we develop a testable expression construct model that incorporates endogenous *Symbiodinium* promoters, terminators, and genes of interest, as well as an internal ribosomal entry site from a *Symbiodinium* virus. Furthermore, we assess the potential for CRISPR/Cas9 genome editing through new analyses of the three currently available *Symbiodinium* genomes. Finally, we discuss how genetic engineering could be applied to enhance the stress tolerance of *Symbiodinium*, and in turn, coral reefs.

Keywords: synthetic biology, genetic engineering, dinoflagellate, *Symbiodinium*, zooxanthellae, coral bleaching

INTRODUCTION

Photosynthetic dinoflagellates are critical primary producers in the aquatic environment, yet, their functional genomics are largely unexplored (Leggat et al., 2011; Murray et al., 2016). *Symbiodinium* is considered one of the most important dinoflagellate genera given its role as the essential photosymbiont of many tropical reef invertebrates, notably reef-building corals (Trench and Blank, 1987). Provision of photosynthetically derived metabolites from *Symbiodinium* to the coral host drives coral calcification and growth that forms the foundation of coral reef ecosystems (Muscatine and Porter, 1977; Muscatine, 1990; Kirk and Weis, 2016). Thermal and light stress

cause photosynthetic dysfunction of *Symbiodinium* and increased leakage of harmful reactive oxygen species from their cells, a process considered largely responsible for the dissociation of *Symbiodinium* from corals characterized as “coral bleaching” (Warner et al., 1999; Suggett et al., 2008; Weis, 2008; Levin et al., 2016). *Symbiodinium* has therefore become established as a major focus for research globally, and in effect, a model genus for dinoflagellates.

Dinoflagellates evolved an estimated 520 million years ago (Moldowan and Talyzina, 1998) and exhibit substantial evolutionary divergence from model eukaryotic organisms including other microalgae such as *Chlamydomonas* and diatoms (Shoguchi et al., 2013). Consequently, dinoflagellates possess unusual biological features that have hindered research progress, such as some of the largest known nuclear genomes (1.5–112 Gbp, typically exceeding the size of the human haploid genome), permanently condensed liquid-crystalline chromosomes, trans-splicing of polycistronic mRNAs, and plastid genomes that are divided up into minicircles (Shoguchi et al., 2013; Zhang et al., 2013; Lin et al., 2015; Murray et al., 2016). The *Symbiodinium* genus evolved an estimated 50 million years ago and is highly diverse, containing nine major evolutionary lineages or “clades” (A–I; Coffroth and Santos, 2005; Pochon et al., 2006; Pochon and Gates, 2010) with hundreds of genetically distinct “types/sub-clades” considered to be different species¹ (Tonk et al., 2013). Genetic factors that promote differences in stress tolerance between *Symbiodinium* variants (both inter- and intra-specific) strongly influence coral gene expression and bleaching susceptibility (Berkelmans and van Oppen, 2006; DeSalvo et al., 2010; Yuyama et al., 2012; Levin et al., 2016). However, the capacity to fully explore *Symbiodinium* genetics is currently restricted by a lack of genetic engineering capability. Genetic engineering has been central to the study of gene function and phenotypic enhancement in organisms ranging from microbes to mammals and a key platform for socioeconomic industries and biotechnologies; yet only two cases of transgene expression in *Symbiodinium* have ever been validated (ten Lohuis and Miller, 1998; Ortiz-Matamoros et al., 2015a).

In 1998, a type A1 strain was transformed at very low efficiencies using silicon carbide whiskers with plasmids encoding expression constructs with plant, plant-viral, and agrobacterial promoters (nos, CaMV 35S, and p1'2') to drive transcription of antibiotic resistance genes (*npII* and *hptII*) and a reporter gene (*GUS*) (ten Lohuis and Miller, 1998); however, these results have yet to be reproduced. It was not until 2015 that another case of transgene expression in *Symbiodinium* was reported (Ortiz-Matamoros et al., 2015b). Plasmids encoding expression constructs with plant and plant-viral promoters (nos and double CaMV 35S) to drive transcription of a herbicide resistance gene (*bar*) and a reporter gene (*GFP*) were introduced to type A1, B1, and F1 strains using glass beads. Whilst cells transiently exhibited improved herbicide resistance and suggestive *GFP* signal, transformations were not validated through DNA, RNA, or protein analysis (Ortiz-Matamoros et al.,

2015b). Further transformation of these strains was attempted using *Agrobacterium* carrying plasmids with the same expression constructs, but the transformants were transient and unable to divide (Ortiz-Matamoros et al., 2015a). Of these studies, none attempted manipulation of ecologically relevant genes thereby limiting new insight gained into *Symbiodinium* biology.

Therefore, in an attempt to overcome the bottleneck that has become established in transforming *Symbiodinium* (and other dinoflagellates), we recommend a new approach that capitalizes on the recent surge in “omics” breakthroughs (Figure 1). By evaluating the rapidly increasing supply of next-generation sequencing (NGS) data, we propose a genetic engineering framework for *Symbiodinium* that may markedly advance our understanding of these important dinoflagellates. Furthermore, genetic manipulation of *Symbiodinium* in order to reduce coral bleaching has been hypothesized as a strategy to facilitate coral management as reefs continue to rapidly deteriorate under climate change (van Oppen et al., 2017). Combatting the impacts of climate change and conserving marine organisms are both key goals for sustainable development set forth by the United Nations². Thus, we believe genetic engineering of *Symbiodinium* may open a novel avenue to achieve these goals by protecting corals from climate change.

TAILORING A GENETIC ENGINEERING FRAMEWORK FOR *Symbiodinium*

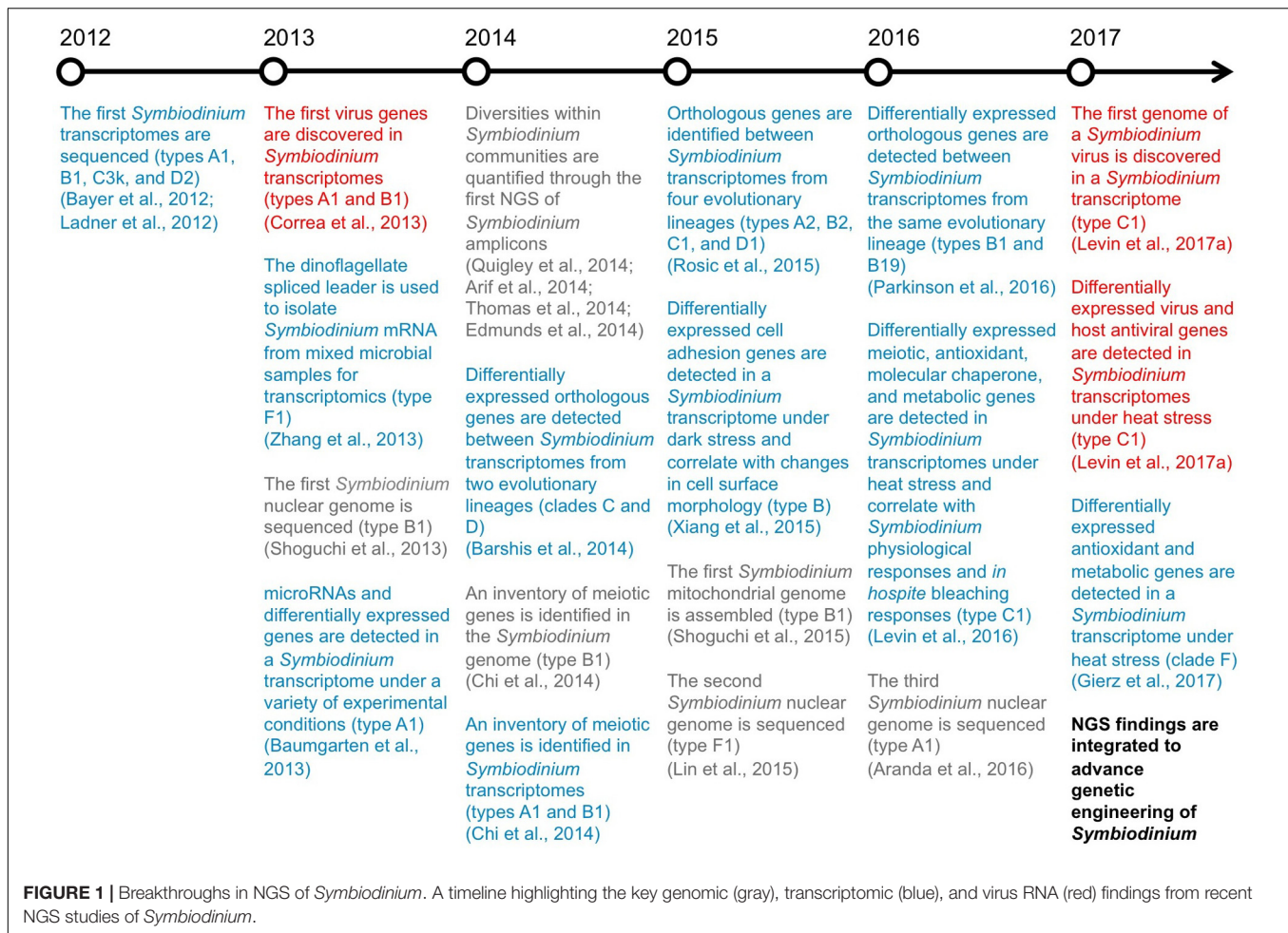
Fundamental components of *Symbiodinium* biology have recently been uncovered through a boom in NGS (Figure 1), particularly the assembly of the first *Symbiodinium* genomes and transcriptomes, direct correlation between *Symbiodinium* transcriptional and physiological states, and discovery of genes from viruses actively infecting *Symbiodinium* cells. Furthermore, NGS of *Symbiodinium* has revealed genetic elements that may allow for transformation of *Symbiodinium*. In the following sections, we detail how unique *Symbiodinium* promoters, specific *Symbiodinium* genes underpinning important phenotypes, and a viral internal ribosomal entry site recognized by *Symbiodinium* ribosomes could be integrated to build expression constructs for *Symbiodinium*.

TRANSCRIPTIONAL PROMOTERS AND TERMINATORS

Currently, dinoflagellate nuclear genome assemblies are all from the genus *Symbiodinium* (types A1, B1, and F1; Shoguchi et al., 2013; Lin et al., 2015; Aranda et al., 2016), emphasizing the importance of *Symbiodinium* to dinoflagellate research. The assemblies have revealed the immense size of *Symbiodinium* genomes with 36,850–49,109 genes, unidirectional gene orientation, prevalent gene tandem arrays, microRNAs along with putative gene targets, and unique promoter architecture (Shoguchi et al., 2013; Lin et al., 2015; Aranda et al., 2016).

¹<http://www.symbiogbr.org/>

²<https://sustainabledevelopment.un.org/?menu=1300>, last accessed March 2017.

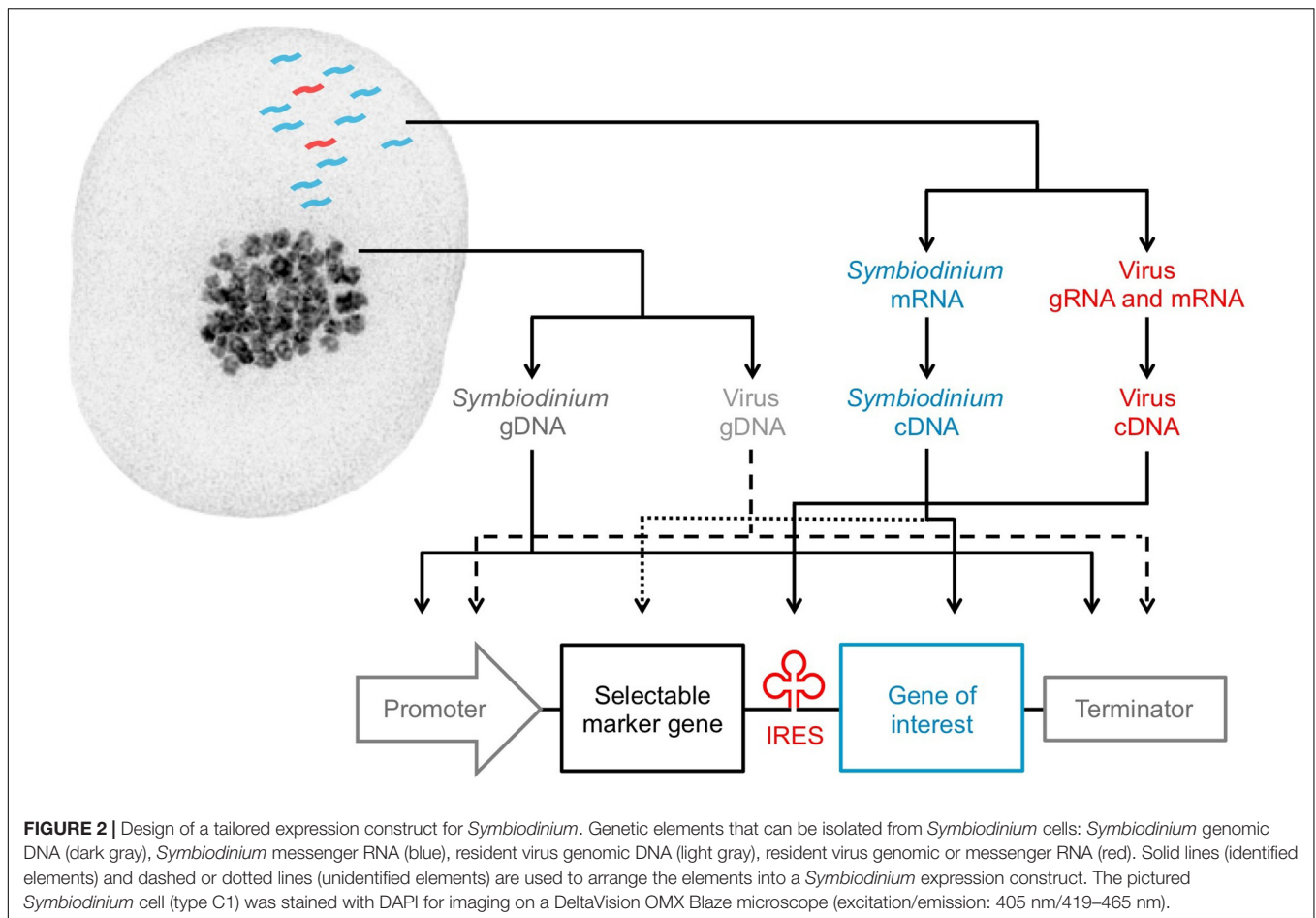


Rather than the traditional TATA-box of eukaryotic promoters, *Symbiodinium* promoters appear to have a TTTT-box that is followed by a unique transcription start site (YYANWYY), branch point (YTNAVY), and acceptor for the dinoflagellate spliced leader (AG) (Lin et al., 2015). Additionally, instead of the typical eukaryotic polyadenylation signal AAUAAA, dinoflagellate terminators use AAAAG/C (Bachvaroff and Place, 2008). Hence, utilization of endogenous *Symbiodinium* promoters and terminators (as opposed to promoters and terminators from other organisms) would likely improve expression and stability of transgenes introduced into *Symbiodinium*. By chance, the CAMV 35S (plant-viral) promoter happens to contain all of the described *Symbiodinium* promoter elements, and the CAMV 35S (plant-viral) and nos (plant) terminators both contain the dinoflagellate polyadenylation signal; this may have contributed to their ability to drive transgene expression in *Symbiodinium* previously (ten Lohuis and Miller, 1998; Ortiz-Matamoros et al., 2015a).

Recent transcriptomic studies have identified highly expressed *Symbiodinium* nuclear genes that can be genome-mapped to uncover strong, endogenous promoters and their corresponding terminators. These promoters and terminators can be isolated from purified genomic DNA (gDNA) through PCR and

incorporated into custom DNA expression constructs for *Symbiodinium* (Figure 2). Among the most highly expressed transcripts in *Symbiodinium* transcriptomes are genes for peridinin-chlorophyll *a*-binding protein, carotenochlorophyll *a-c*-binding protein, major basic nuclear protein 2, dinoflagellate viral nucleoprotein, and glyceraldehyde-3-phosphate dehydrogenase (Baumgarten et al., 2013; Levin et al., 2016; Parkinson et al., 2016); though all are multi-copy genes (Shoguchi et al., 2013; Lin et al., 2015; Aranda et al., 2016). Ideally, highly expressed nuclear genes chosen for promoter selection should not have high copy numbers, as their expression levels may largely be due to prevalence in the genome rather than strong promoters. Constitutively expressed nuclear genes are also desirable for selection of promoters that drive consistent transcription regardless of experimental conditions, and thus, drive reliable transgene expression.

To illustrate this approach of *Symbiodinium* promoter selection, we examined NGS data from a type A1 *Symbiodinium* strain for which the nuclear genome has been recently sequenced (Aranda et al., 2016) and the transcriptional responses to various conditions (temperatures, ionic stress, dark stress, and contrasting circadian rhythm time points) have been determined (Baumgarten et al., 2013). Locus 144 and Locus 1768 in the



type A1 transcriptome, a subunit of a large neutral amino acids transporter and a putative ATP-binding cassette transporter gene, both show high expression across all conditions (average expression in the top 2% of all genes; Baumgarten et al., 2013) and map tightly to the type A1 genome scaffolds 710 and 484, respectively. No significant open reading frames are found > 5 kb up- or down-stream of either gene, confirming that they are not part of tandem arrays. For each gene, all *Symbiodinium* promoter elements are within 1 kb of the start codon, and the dinoflagellate polyadenylation signal is found ~300 bp after the stop codon. These promoter and terminator regions could therefore be isolated and utilized to drive high and consistent expression of transgenes in a *Symbiodinium* expression construct.

GENES OF INTEREST

Recent transcriptomic studies have been fundamental in the discovery of *Symbiodinium* nuclear genes that underpin phenotypic traits, such as those related to cell adhesion (e.g., *GspB*, *Svep1*, *Slap1*; Xiang et al., 2015), sexual reproduction (e.g., *Msh4*, *Msh5*, *Spo11-2*; Chi et al., 2014; Levin et al., 2016; Gierz et al., 2017), antiviral response (e.g., *Birc3*, *Ns1bp*, *Ifih1*; Levin et al., 2017a), and antioxidant activity/thermal tolerance

(e.g., *Fe-sod*, *Mn-sod*, *Pxrd*, *Hsp70*; Levin et al., 2016; Gierz et al., 2017). *Symbiodinium* antioxidant genes are of particular interests because of their potential role in defining bleaching susceptibility of the coral host (Krueger et al., 2015; Levin et al., 2016). For instance, iron-type superoxide dismutase (*Fe-sod*) genes are believed to minimize thermally induced oxidative damage to photosynthetic apparatuses and leakage of harmful reactive oxygen species from type C1 *Symbiodinium* cells—determinants of coral bleaching (Weis, 2008); however, these genes are not expressed at detectable levels in all *Symbiodinium* variants (Krueger et al., 2015; Levin et al., 2016). A *Fe-sod* gene could therefore be inserted after a strong *Symbiodinium* promoter in an expression construct to drive its over-expression for evaluation of its phenotypic influence on *Symbiodinium*. Endogenous genes of interest should be isolated through PCR of complementary DNA (cDNA) reverse transcribed from purified mRNA, since gDNA introns may prevent proper expression in constructs (Figure 2).

Expression of exogenous genes of interest in *Symbiodinium* could also greatly advance investigations of ecological processes central to coral reef health. For instance, documenting competition between *Symbiodinium* types, transmission and acquisition of *Symbiodinium* types by the coral host, and shuffling of *Symbiodinium* types within host tissues

(Toller et al., 2001; van Oppen et al., 2001; Little et al., 2004; Berkelmans and van Oppen, 2006; Byler et al., 2013; Boulotte et al., 2016) is currently reliant upon sequencing since it is not possible to visually differentiate many types. As a result, studies have been restricted to low temporal and spatial resolution relative to real-time imaging. Instead, the ability to color-code *Symbiodinium* types through genetic transformation with various fluorescent proteins could illuminate these phenomena by enabling real-time imaging for visually differentiating types. Additionally, tagging endogenous genes of interest through fluorescent protein fusions would permit imaging of protein localization within *Symbiodinium* cells and potential protein secretion out of *Symbiodinium* cells (Xiang et al., 2015). When selecting appropriate fluorescent proteins, it will be imperative to consider the extreme autofluorescence of *Symbiodinium* (Shaner et al., 2005); for example, venus (excitation/emission: 515/528 nm), tdTomato (excitation/emission: 554/581 nm), and mCherry (excitation/emission: 587/610 nm) are promising candidates as their fluorescence properties are off-peak of the *Symbiodinium* excitation and emission spectra (Hennige et al., 2009; Jiang et al., 2012). Finally, codon optimization may be necessary for optimal exogenous gene expression in *Symbiodinium* since codon usage of *Symbiodinium* genes can be divergent from foreign genes (Levin et al., 2017a) and even between *Symbiodinium* nuclear and minicircle genes (Bayer et al., 2012).

SELECTABLE MARKER GENES

Although antibiotics have previously been used to select transformed *Symbiodinium* (ten Lohuis and Miller, 1998; Ortiz-Matamoros et al., 2015a), their use is problematic for two main reasons. Firstly, eliminating wild-type *Symbiodinium* in culture requires high concentrations of antibiotics (e.g., 3 mg/ml of G418 or hygromycin; ten Lohuis and Miller, 1998), making experimentation and long-term maintenance of transformed cell lines extremely costly. It is also important to note that natural antibiotic resistances are not uniform across all strains (Supplementary Table 1), so dosage curves are necessary before conducting transformation trials. Secondly, dinoflagellates including *Symbiodinium* require symbiotic bacteria to grow optimally (Alavi et al., 2001; Croft et al., 2005; Miller and Belas, 2006; Ritchie, 2012). Since eukaryotic antibiotics can also be toxic to prokaryotes (Gonzalez et al., 1978; Colanduoni and Villafranca, 1986; Pline et al., 2001; Vicens and Westhof, 2003), bacterial communities in *Symbiodinium* cultures are removed during antibiotic selection.

To preserve symbiotic bacteria, alternatives to antibiotic selection markers should be considered, such as genes that provide growth advantages under specific conditions by increasing pathogen resistance, increasing thermal tolerance, or allowing for utilization of non-metabolized carbohydrates (Breyer et al., 2014). The precise functions of these alternative marker genes (e.g., phosphomannose isomerase) are well defined and shown to be applicable to many photosynthetic species (Stoykova and Stoeva-Popova, 2011), though their

compatibilities with dinoflagellates are unknown. Discovery of endogenous selectable markers should therefore also be pursued. Recent *Symbiodinium* transcriptomic studies have uncovered genes involved in selection-relevant phenotypes like photosynthetic ability at unique light regimes (Parkinson et al., 2016) or tolerance to increased temperature regimes (Levin et al., 2016). These *Symbiodinium* genes could first be expressed in more easily transformed microalgae like *Chlamydomonas* and diatoms to gauge the potential for their up-regulation to grant a significant selectable advantage under specific conditions.

VIRAL ELEMENTS

Viral promoters and terminators, internal ribosome entry sites (IRES), and 2A peptides are staple regulatory elements incorporated in expression constructs since they have evolved to be recognized by eukaryotic machinery for efficient and stable foreign gene expression (Benfey and Chua, 1990; Martínez-Salas, 1999; Levin et al., 2014). *Symbiodinium* transcriptomics have led to the discovery of genes, as well as an entire RNA genome, from novel eukaryotic viruses that infect *Symbiodinium* (Correa et al., 2013; Levin et al., 2017a). A putative viral IRES, which allows cap-independent translation to produce separate proteins from one mRNA transcript, was found between the two open reading frames in the RNA genome of the +ssRNA virus infecting type C1 *Symbiodinium* (GenBank accession: KX538960 and KX787934; Levin et al., 2017a). The +ssRNA virus transcripts were extremely abundant in a type C1 *Symbiodinium* transcriptome (Levin et al., 2017a), and such rampant +ssRNA virus replication indicates that *Symbiodinium* ribosomes have high affinity to this IRES.

IRES sequences enable the creation of polycistronic constructs transcriptionally controlled by a single promoter (Martínez-Salas, 1999). By permitting simultaneous expression of two independent proteins from one mRNA, a bicistronic construct can achieve long-term expression of a gene of interest because the gene of interest is transcriptionally fused to the selectable marker gene (Gurtu et al., 1996; **Figure 2**). Conversely, in monocistronic constructs, the selectable marker gene often maintains expression, while the gene of interest becomes transcriptionally repressed over time if it does not increase fitness of the cell (Allera-Moreau et al., 2007). Therefore, the IRES from the *Symbiodinium* +ssRNA virus is a valuable viral element that is recognized by *Symbiodinium* ribosomes and may improve the stability of transgene expression in *Symbiodinium*. Moving forward, NGS data of viruses in *Symbiodinium* cultures (Weynberg et al., 2017) and the coral holobiont (Weynberg et al., 2014; Correa et al., 2016) should be mined for promoter, terminator, and other regulatory elements from *Symbiodinium* viruses, given the proven benefits of viral elements to genetic engineering. Once assembled, the *Symbiodinium* expression construct (**Figure 2**) can be combined with the backbone of a standard cloning plasmid; added into an artificial, replicating minicircle (Nehlsen et al., 2006; Karas et al., 2015); or serve as a repair template for CRISPR/Cas9 genome editing (Cong et al., 2013).

CRISPR/Cas9 GENOME EDITING AND *Symbiodinium*

Within the past 5 years, CRISPR/Cas9 has revolutionized genome editing by allowing precise changes to be made to target sites in the genome (Cong et al., 2013; Baek et al., 2016; Nymark et al., 2016). In short, a single guide RNA (sgRNA) is designed to recruit the Cas9 endonuclease protein and to match a specific, desired target site in the genome that must be immediately followed by a protospacer adjacent motif (PAM) sequence (5'-NGG-3'). Once complexed with Cas9, the sgRNA guides Cas9 to the target genome site. Cas9 then interacts with the PAM sequence and creates a double-strand break in the target site. The cell can either repair the double stranded break through non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Ran et al., 2013). NHEJ genome editing arises from introduction of a random mutation/insertion/deletion when the broken ends of DNA are directly ligated, which can cause the target gene to be knocked out (i.e., non-functional). Gene knockout provides insight into the role and criticality of a gene by assessing the effect of its absence. Alternatively, HDR genome editing uses a repair template flanked by 5' and 3' homologous arm sequences that match the up- and down-stream regions of the double-strand break. The repair template can be designed for gene knockout, introduction of a specific mutation/insertion/deletion, or genomic integration of a transgene(s)/entire expression construct (Ran et al., 2013).

Symbiodinium exhibits an asexual haploid vegetative stage (Santos and Coffroth, 2003) with sister chromatids developing in S-phase of the cell cycle (Watrin and Legagneux, 2003), but HDR has yet to be directly observed in *Symbiodinium*. Therefore, CRISPR/Cas9 genome editing of *Symbiodinium* may be restricted to NHEJ. *Ku70*, *Ku80*, and DNA ligase IV (genes central to NHEJ; Chu et al., 2015) are all expressed in *Symbiodinium* transcriptomes (Levin et al., 2016). That said, some evidence does suggest *Symbiodinium* can enter a transient sexual diploid stage (Chi et al., 2014; Wilkinson et al., 2015; Levin et al., 2016), which has been documented in other dinoflagellates (Figueroa et al., 2015). In yeast, ploidy shifts the dominant double-stranded break repair mechanism—diploid cells favor HDR, while haploid cells favor NHEJ (Lee et al., 1999). Moreover, genes specific to meiosis, a process during which HDR occurs (Thacker and Keeney, 2016), have been found in *Symbiodinium* genomes and transcriptomes (Chi et al., 2014; Lin et al., 2015; Rosic et al., 2015; Levin et al., 2016). *Msh4*, *Msh5*, and *Spo11-2* are all highly up-regulated at elevated temperatures (Levin et al., 2016), suggesting that HDR pathways in *Symbiodinium* are activated. *Brca2*, a gene that controls HDR (Holloman, 2011), is likewise up-regulated in heat stressed *Symbiodinium* (SM population: TR74441| c0_g1; MI population: TR63986| c0_g1; Levin et al., 2016). Hence, the potential for genomic integration of transgenes through HDR may improve if *Symbiodinium* are pre-stressed. HDR in *Symbiodinium* may also be increased by suppression of *Ku70*, *Ku80*, or DNA ligase IV (Chu et al., 2015).

The permanently condensed chromosomes of *Symbiodinium* could present an obstacle for CRISPR/Cas9 genome editing by

possibly limiting access of sgRNAs to certain target sites. An additional challenge for genome editing is the abundance of multi-copy genes in the large *Symbiodinium* genomes. Gene redundancy can prevent knockout of gene function since the CRISPR/Cas9 system is not 100% efficient, meaning uncleaved functional gene copies can remain. Additionally, CRISPR/Cas9 targeting of genes with high copy numbers has been found to decrease cell proliferation and survival likely due to an increased frequency of DNA damage events (Aguirre et al., 2016). Also, design of sgRNAs requires a sequenced genome, but only three *Symbiodinium* genomes—each from a separate evolutionary lineage—are currently available.

As a first step to overcome some of these limitations, we analyzed the three published *Symbiodinium* genomes (types A1, B1, and F1; Shoguchi et al., 2013; Lin et al., 2015; Aranda et al., 2016) to identify conserved single copy genes. We then predicted a target site in each conserved gene with high sgRNA efficiency and specificity across the genomes (Supplementary Materials and Methods). Conserved target sites may permit CRISPR/Cas9 genome editing of *Symbiodinium* types that have yet to be sequenced. Our analysis revealed 1792 conserved single copy orthologs, 261 of which have an optimal target site compatible with all genomes (Supplementary Dataset 1a). The 261 single copy orthologs for CRISPR/Cas9 genome editing were enriched for a wide array of functional gene groups of interest, including cellular components for photosynthesis and biological pathways for oxidation-reduction and for response to UV-B (Supplementary Figure 1 and Supplementary Tables 2–4). Knockout of these genes would critically improve our understanding of *Symbiodinium* gene function, and if HDR is present in *Symbiodinium*, these sgRNA target sites could also be used to introduce genes of interest or entire *Symbiodinium* expression constructs into the genome. Furthermore, we identified sgRNA target sites in the type A1 genome scaffolds 710 and 484 (Aranda et al., 2016) immediately downstream from the potentially strong, constitutive *Symbiodinium* promoters discussed earlier (Supplementary Dataset 1b). Assuming HDR, reporter genes such as fluorescent proteins could be introduced at these sites to measure promoter activity.

The CRISPR/Cas9 system can be carried by plasmids that contain expression constructs for the Cas9, sgRNA, and in the case of HDR, the repair template with homologous arms. Target site cleavage is improved by increased CRISPR/Cas9 construct expression (Hsu et al., 2013), so strong endogenous promoters and terminators from *Symbiodinium* discussed earlier could be employed to drive transcription of Cas9 by *Symbiodinium*. However, transcription of sgRNAs requires RNA polymerase III (*Pol III*) rather than RNA polymerase II. Therefore, promoters specifically recognized by *Pol III* (e.g., promoter of the U6 snRNA gene) are needed. Such promoters have been isolated from other eukaryotes for sgRNA transcription; but, as discussed earlier, they contain motifs (e.g., TATA-box) that *Symbiodinium* lack (Goomer and Kunkel, 1992; Clarke et al., 2013). In *Symbiodinium*, 26 U6 snRNA gene copies have been identified (see Supplementary Table 5 in Shoguchi et al., 2013), one of which is unusually located in a cluster with U1, U2, U4,

U5, 5S, and spliced leader snRNA genes (type B1 genome scaffold 8131; Shoguchi et al., 2013). Thus, genomic sequences found upstream and downstream of these *Symbiodinium* U6 snRNA genes could be isolated and trialed in sgRNA expression constructs as potential promoters and terminators recognized by *Symbiodinium Pol III*. Alternatively, the CRISPR/Cas9 system can be introduced to cells as pre-complexed sgRNA and purified Cas9 protein, which can achieve higher genome editing specificity by ~10-fold compared to CRISPR/Cas9 plasmids and also removes the need to optimize Cas9 codon usage or to find appropriate promoters that will express Cas9 or sgRNAs (Zuris et al., 2015).

INTRACELLULAR DELIVERY OF CONSTRUCTS AND COMPLEXES

Verified delivery of expression constructs into *Symbiodinium* was previously achieved using silicon carbide whiskers, which yielded very few transformants (ten Lohuis and Miller, 1998), and with *Agrobacterium*, which produced transient transformants that were unable to divide (Ortiz-Matamoros et al., 2015a). Low efficiency foreign DNA delivery may be due to obstruction by the thick, multilayer *Symbiodinium* cell covering comprised of an external polysaccharide or glycoprotein layer atop an internal cell wall (thecal plates and the pellicle) then finally the plasma membrane (Markell et al., 1992; Wakefield et al., 2000). To overcome this barrier, methods including high-voltage electroporation, bioballistics, microinjection, and viral transduction should be trialed. Continued exploration into *Symbiodinium* viruses may facilitate development of a compatible transduction system. Additionally, the first method to produce viable *Symbiodinium* protoplasts (cells with their cell wall removed) was developed (Levin et al., 2017b). Protoplasts have been instrumental in genetic manipulation of cell-walled organisms through somatic hybridization as well as by allowing for alternate DNA delivery methods (Davey et al., 2005). Protoplast-dependent methods such as polyethylene glycol-mediated transformation (Mathur and Koncz, 1998) and liposome-mediated transformation (Caboche, 1990) may improve efficiency of construct delivery into *Symbiodinium*. Cell walls also serve as a barrier to RNA/protein complexes like pre-complexed sgRNA and Cas9 protein. Thus, genome editing of *Symbiodinium* with pre-complexed sgRNA and Cas9 protein may require the use of protoplasts (Woo et al., 2015). Polyethylene glycol-mediated transformation (Woo et al., 2015), cationic lipid transformation (Zuris et al., 2015), and electroporation (Baek et al., 2016) have all been used to effectively deliver pre-complexed sgRNA and Cas9 protein through cell membranes of other eukaryotes that lacked cell walls.

CAN WE REDUCE CORAL BLEACHING WITH GENETICALLY ENHANCED *Symbiodinium*?

Coral reefs are the most diverse marine habitat per unit area (Reaka-Kudla et al., 1996; Knowlton et al., 2010) and provide

world economies with nearly US\$30 billion in net benefits from goods and services annually (Cesar et al., 2003). Climate change impact models predict that most reefs will be severely damaged or lost in this century unless immediate protection efforts are made (Hoegh-Guldberg et al., 2007; Pandolfi et al., 2011; Mora et al., 2016; Hughes et al., 2017) prompting calls for the development of novel mitigation and restoration approaches (Rinkevich, 2014; van Oppen et al., 2015, 2017; Piaggio et al., 2016). Exceptional genetic variability naturally exists within the genus *Symbiodinium*, suggesting that seeding vulnerable corals with more climate-change tolerant *Symbiodinium* variants could provide a means to reduce bleaching susceptibility of corals (van Oppen et al., 2015). Although, uptake of non-native *Symbiodinium* variants by corals may not be widely achievable since many coral species only associate with specific *Symbiodinium* types (LaJeunesse et al., 2004). Furthermore, shifts from innately less stress tolerant *Symbiodinium* types to more stress tolerant *Symbiodinium* types (e.g., from type C2 to D) can have negative impacts on a number of coral fitness traits including growth and fecundity (Little et al., 2004; Jones and Berkelmans, 2011).

Environmental bioengineering is an alternative strategy to safeguard against climate change (Solé, 2015; Piaggio et al., 2016). Microalgae, such as *Symbiodinium*, are clear and promising candidates for genetic engineering with the aim of regaining and preserving ecosystem-climate homeostasis (Solé, 2015) because they can significantly influence the health of entire ecosystems (Berkelmans and van Oppen, 2006; Kirk and Weis, 2016; Murray et al., 2016). Genetic engineering to increase stress tolerance of the *Symbiodinium* variants that are naturally harbored by at-risk corals holds potential to reduce bleaching susceptibility without negatively impacting the fitness of the coral host since existing *Symbiodinium*-coral partnerships would be preserved. *Fe-sod*, *Mn-sod Prxd*, and *Hsp70* genes from *Symbiodinium* (Levin et al., 2016; Gierz et al., 2017; Goyen et al., 2017) are standout candidates whose engineered up-regulation may enhance thermal and bleaching tolerance by reducing heat-induced oxidative damage, but thorough evaluation of how this artificial up-regulation contributes to long term fitness and the *Symbiodinium*-coral symbiosis would be mandatory.

Application of genetic engineering to support environmental management practices has been gaining momentum. Notably, sterile male mosquitoes have been engineered to control mosquito-borne diseases (Gabrieli et al., 2014). Field releases of the sterile males significantly reduced wild mosquito populations, supporting their value to disease control (Harris et al., 2012). Similarly, fungus-resistance has been engineered in American chestnut trees in order to restore the natural population that was nearly eradicated from the spread of a foreign fungus. Introduction of these transgenic trees into the wild may receive federal approval in just the next few years, which would make them the first threatened plant species to be restored through genetic engineering (Jacobs et al., 2013; Powell, 2014).

Considering the great promise shown by genetic engineering-based approaches to promote environmental health (Jacobs et al., 2013; Powell, 2014) and human health (Paine et al., 2005; Harris et al., 2012; Gabrieli et al., 2014), as well as to sustain

food security (Schroeder et al., 2013), it is logical for genetic engineering to be proposed as an important component of the growing repertoire of forward-looking coral reef management approaches (van Oppen et al., 2015; Piaggio et al., 2016). Due to the urgent need to protect coral reefs from climate change, the *Symbiodinium* research community must commit to an all-hands-on-deck attitude to achieve and extensively test genetic enhancement of *Symbiodinium* and other novel reef restoration strategies in the laboratory setting. In parallel, comprehensive cost-benefit-risk evaluation of the potential ecological and socioeconomic impacts from implementation of such strategies in the natural environment must be exhaustive before field-based trials are initiated. Additionally, transparent dialogs with policy makers, coral reef managers, and the general public need to be initiated now to begin the process of education and public acceptance of genetic engineering approaches for coral reef mitigation and restoration.

As we have discussed here, recent NGS breakthroughs have revealed natural genetic elements of *Symbiodinium* and their viruses (Figure 1). Based on these discoveries, we have developed a tailored genetic engineering framework for *Symbiodinium* based on empirical data that may also be applicable to other dinoflagellate genera. In doing so, we have opened a new prospective avenue to decode *Symbiodinium* functional genomics that may ultimately allow for engineering increased stress tolerance of *Symbiodinium* to reduce coral bleaching.

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AUTHOR CONTRIBUTIONS

RL conceived the manuscript concept, analyzed NGS data, and wrote the manuscript. CRV analyzed NGS data and critically edited the theory and writing of the manuscript. SA analyzed NGS data. PS, DS, and MvO critically edited the theory and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.01220/full#supplementary-material>

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The reviewer AWL declared a shared affiliation, though no other collaboration, with one of the authors DS to the handling Editor, who ensured that the process nevertheless met the standards of a fair and objective review.

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