

Progress of CRISPR-Cas Based Genome Editing in Photosynthetic Microbes

Mihris Ibnu Saleem Naduthodi, Maria J. Barbosa, and John van der Oost*

The carbon footprint caused by unsustainable development and its environmental and economic impact has become a major concern in the past few decades. Photosynthetic microbes such as microalgae and cyanobacteria are capable of accumulating value-added compounds from carbon dioxide, and have been regarded as environmentally friendly alternatives to reduce the usage of fossil fuels, thereby contributing to reducing the carbon footprint. This light-driven generation of green chemicals and biofuels has triggered the research for metabolic engineering of these photosynthetic microbes. CRISPR-Cas systems are successfully implemented across a wide range of prokaryotic and eukaryotic species for efficient genome editing. However, the inception of this genome editing tool in microalgal and cyanobacterial species took off rather slowly due to various complications. In this review, we elaborate on the established CRISPR-Cas based genome editing in various microalgal and cyanobacterial species. The complications associated with CRISPR-Cas based genome editing in these species are addressed along with possible strategies to overcome these issues. It is anticipated that in the near future this will result in improving and expanding the microalgal and cyanobacterial genome engineering toolbox.

1. Introduction

The exploitation of non-renewable energy sources to meet the perpetual requirement of increasing human population has resulted in their rapid depletion and a steady rise in price. Moreover, their uncontrolled usage has resulted in an elevated CO₂ concentration in the atmosphere, resulting in global warming and associated problems. In this scenario, it is of utmost importance to find environmentally friendly alternatives

to meet the demands of the developing world. The potential of deploying photosynthetic microbes such as microalgae and cyanobacteria for production of next generation fuels is being studied extensively.^[1] The ability to fix CO₂ and convert it into value added compounds without competing with food and feed crops make these green microbes promising biofuel producing organisms from an environmental perspective.^[2] However, studies on microalgae to exploit their complete potential for commercial application has not proceeded fast mainly because of the lack of industrial strains. Efficient genome editing tools for microalgae are still lacking. Conventional homologous recombination-based gene editing was reported in genera like *Nannochloropsis* and *Ostreococcus*. The Zinc Finger Nucleases (ZFN) were applied in *Chlamydomonas reinhardtii* for achieving targeted gene editing, while Transcription Activator Like Effector Nucleases (TALEN) were implemented in *Phaeodactylum tricoratum*.^[3] Nevertheless, the labor intensiveness, complexity and high cost for

application was a bottleneck for implementing ZFN and TALEN for genome engineering. Since 2014, CRISPR-Cas based genome editing has been reported in various microalgal species, which will be the prime focus of this review.^[4]

The genome editing of both prokaryotes and eukaryotes has been simplified with the introduction of RNA guided nucleases of CRISPR-Cas systems (Clustered regularly interspaced short palindromic repeats and associated proteins).^[5,6] These endonucleases use short CRISPR-derived RNA guides to target complementary DNA. After recognition of a protospacer adjacent motif (PAM) sequence positioned next to the target sequence, the Cas nuclease (Cas9, Cpf1/Cas12a) introduces double strand DNA breaks (DSBs). Mutating the catalytic active site of Cas9 protein results in a dead Cas9 (dCas9)^[7] that has been implemented for CRISPR interference (CRISPRi) by stably binding, for instance to the promoter region of a target gene, and thereby downregulating the transcription of a target gene.^[7,8] The CRISPR systems are divided into two major classes based on their architecture: class I systems (types I, III, and IV) form multi subunit protein crRNA-binding nuclease complexes, and class II system (type II, V and VI) consist of a single guide-binding nuclease protein.^[9] Initially, the Cas9 from *Streptococcus pyogenes* (SpCas9, archetype of the type II CRISPR-Cas system) and related Cas9 variants have been widely applied for genome

M. I. S. Naduthodi, Prof. J. van der Oost
Laboratory of Microbiology
Wageningen University and Research
Wageningen, Netherlands
E-mail: john.vanderoost@wur.nl

M. I. S. Naduthodi, Dr. M. J. Barbosa
Bioprocess Engineering, AlgaePARC
Wageningen University and Research
Wageningen, Netherlands

© 2018 The Authors. *Biotechnology Journal* Published by Wiley-VCH Verlag GmbH & Co. KGaA. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1002/biot.201700591

engineering. Recently, Cas12a (Cpf1) of the type V system is also gaining global attention for genome editing in various species.^[10–12] Cas12a is an interesting alternative tool for genome engineering as it has distinct features compared to Cas9 (Figure 1), such as (i) Cas12a uses a single crRNA instead of a set of crRNA and trans-activating crRNA (tracrRNA) in Cas9 that is synthetically fused as a single guide RNA (sgRNA) that is at least twice as long as the crRNA guide used by Cas12a, (ii) Cas12a has been demonstrated to catalyze the maturation of its own crRNA which allows for efficient multiplex genome editing,^[12,13] while maturation of crRNA:tracrRNA complex of Cas9 relies on processing by the non-Cas ribonuclease RNase III, (iii) Cas12a uses a T-rich PAM (5'-TTTN-3') upstream the protospacer in contrast to the downstream located G-rich PAM (5'-NGG-3') that is recognized by Cas9,^[11] (iv) Cas12a generates staggered ends with five nucleotide overhangs in the target DNA, compared to the blunt end cleavage by Cas9,^[11,14] (v) Cas12a has a single nuclease domain (RuvC) that cleaves 18–23 base pairs downstream from the PAM-proximal seed sequence, whereas cleavage by the two nuclease domains of Cas9 (RuvC and HNH) occurs within the seed three base pairs upstream its PAM (Figure 1). These mechanistic differences may have important practical advantages, such as directly using small (42–66 nt), commercially-produced crRNA guides, and the potential to perform multiplex genome editing owing to the self-maturation of a precursor crRNA guide. In addition, it has been proposed that the fact that Cas12a cleavage occurs outside its seed region (Figure 1),^[11] may enhance the frequency of HDR as the seed sequence is not destroyed by Non-Homologous End Joining (NHEJ) in eukaryotes, and hence cleavage could continue after NHEJ repair until HDR is successful (substituting essential nucleotides in PAM and/or seed). This review elaborates on the application of CRISPR-Cas based genome engineering in microalgae and cyanobacteria, and provides insights on strategies to enhance efficiency of the CRISPR-Cas tools for generating targeted mutants in these microbes (Table 1).

2. *Chlamydomonas reinhardtii*

The completely sequenced and annotated genome, the genetic accessibility, the ease of generating and screening mutants owing to its haploid nature and decades of research on cellular and molecular level has made *Chlamydomonas reinhardtii* an exceptional model organism in the field of microalgal research.^[15] The first CRISPR-Cas9 based genome editing in microalgae was reported in *C. reinhardtii*.^[4] Plasmid-based delivery of a codon optimized SpCas9 gene and specific sgRNAs to *Chlamydomonas reinhardtii* has resulted in transient expression and targeting restriction sites of various exogenous genes.^[4] The exogenous target gene as well as the cas9 and sgRNA genes were delivered into the host on the same plasmid. After transformation, the target loci were PCR amplified after which the obtained amplicons were subjected to restriction digestion with the enzyme cleaving the target site. Introduction of indels at the target site by NHEJ upon Cas9 cleavage was hypothesized to result in the fragments' resistance to the specific restriction digestion. Sequencing of the fragments resistant to this restriction digestion revealed indels in the region of Cas9 cut



Mihris Naduthodi completed his bachelor degree in Biotechnology and Biochemical Engineering in 2014 from Sree Chitra Thirunal College of Engineering in India. Later, he pursued his masters in cellular and molecular biotechnology from Wageningen University, Netherlands. Realizing the potential of engineered microbes and fascination on the state

of the art genome engineering techniques [CRISPR-Cas systems] led to his MSc thesis in the Bacterial genetics lab under the supervision of Ioannis Mougiakos, Richard van Kranenburg and John van der Oost. Since September 2017, Mihris has been appointed as a PhD student under the guidance of John van der Oost and Maria Barbosa where he works on developing genome editing tools for metabolic engineering of microalgae.



Maria Barbosa is Associate Professor in Microalgae Biotechnology and Director of AlgaePARC (www.AlgaePARC.com) at Wageningen University, the Netherlands. She holds a PhD in Bioprocess Engineering obtained at Wageningen University. She has worked at ETH (Swiss Federal Institute of Technology), Switzerland and at EMBO (European

Molecular Biology Organisation), Germany. She coordinates several large research programs with more than 50 industrial partners and 20 academic partners, covering the entire microalgae production chain. Her scientific interests are on microalgae strain improvement, cultivation and scale up.



John van der Oost obtained his PhD degree at the Free University in Amsterdam. After postdoc positions in Helsinki, Heidelberg and Amsterdam, he moved to Wageningen University in 1995 where he became group leader of the Bacterial Genetics group in the Laboratory of Microbiology. In 2005 John was appointed Professor, in 2013 he was

elected as EMBO member, and in 2017 as member of the Royal Dutch Society for Arts and Sciences (KNAW). Using an NWO VICI grant and two NWO TOP grants, he established a successful research line on prokaryotic anti-viral defence systems (CRISPR-Cas and prokaryotic Argonaute).

site, confirming Cas9 nuclease activity. However, an experiment involving multiple transformations and more than 10^9 cells for targeting an endogenous gene (*FKB12*) by Cas9 resulted in a single mutant colony, demonstrated a rather low efficiency of

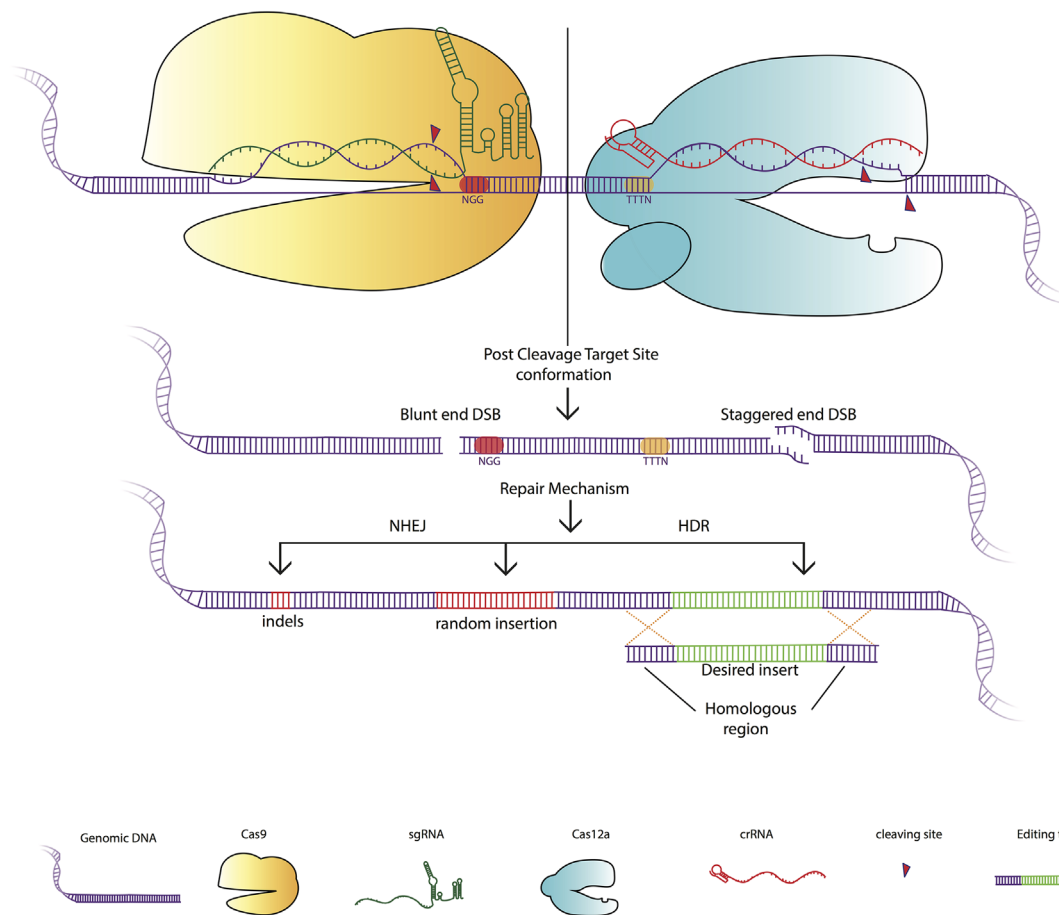


Figure 1. Schematic representation of DSB generation by Cas9 and Cas12a, and repair mechanisms present in eukaryotes for restoring the DSB. The Cas9 causes a blunt end DSB 3 base pairs downstream the PAM 5'NGG/3 and Cas12a introduce staggered end DSB 18 and 23 base pairs away from the PAM 5'TTTN/3 on targeting and non-targeting strands respectively. NHEJ and HDR based repair mechanisms are implemented by eukaryotic cells to repair the DSBs, where indels and random insertions at repair site by NHEJ results in error prone repair, while HDR repair mechanism is results in flawless integration or deletion of target site and provides possibilities for errorless targeted genome editing.

this approach for generating frame-shift mutants. The cytotoxicity of Cas9 in *C. reinhardtii* was inferred for this problem as Western blot analysis failed to detect even minute levels of either Cas9 or dCas9.^[4] Recently, Jiang et al. managed to enhance the efficiency of plasmid-based Cas9 activity in *C. reinhardtii* by implementing a hybrid version of Cas9.^[16] In this work, a gene-within-a-gene approach was used in which an artificial intron sequence harboring a sgRNA was incorporated into the Cas9 coding sequence. The activity of this system was validated by restoring the abolished reading frame in an exogenously supplied mutant antibiotic resistance gene. Indeed, some recombinants gained the capacity to grow on plates with the corresponding antibiotic, indicating Cas9 cleavage and repair by non-homologous end joining (NHEJ).^[16] The targeting of the hybrid Cas9 system was reported to be 50 times more efficient compared to the previous version, as targeting the same gene with this approach yielded 13 mutants among 4×10^8 cells transformed.^[4,16] However, the observation that none of the mutants obtained by the hybrid-Cas9 approach harbored an intact Cas9 gene, strongly suggested toxicity of Cas9.^[16] In the same study, it has also been tested to combine homologous

recombination and Cas9 nuclease activity to achieve precise, error-less gene editing. The transformation with the hybrid-Cas9 and short single stranded DNA repair fragments with single nucleotide mismatches at the Cas9 target site resulted in an average of six correct mutants upon when two different genes were targeted independently, whereas no mutants were obtained when the homologous ssDNA was transformed alone.^[16] This relatively low homologous recombination frequencies in *C. reinhardtii* (in the absence of Cas9) were in agreement with earlier observations.^[17] Also, the induction of DSBs has been reported to enhance the homologous recombination efficiency in various eukaryotes which could have occurred in *C. reinhardtii* facilitating the formation of these six expected mutants.^[6,18]

The plasmid-based expression of two Cas9 variants (spCas9 from *Streptococcus pyogenes* and saCas9 from *Staphylococcus aureus*) in *C. reinhardtii* and their efficiency in producing indel mutations was compared by Greiner et al.^[19] In the experiment targeting the *PSY1* gene by both Cas9 variants independently, application of SaCas9 yielded 9% mutants among the entire antibiotic resistant transformants obtained, outperforming SpCas9 which yielded 3% mutants. The efficiency of targeting

Table 1. Overview of the native genes edited in Photosynthetic microbes using CRISPR-Cas systems.

Species	Strategy for Cas protein delivery	Outcome of the target gene	Modification at the target site	Target genes	Ref.
<i>Chlamydomonas reinhardtii</i>	Plasmid	Knockout	Indels	<i>FKB12, PSY1, Chr2, COP1/2, COPS, PHOT</i>	[7,34]
			HDR	<i>ARG7, ALS</i>	[28]
	RNP	Knockout	Indels	<i>MAA7, CpSRP43, ChIM, CpFTSY, ZEP, PHT7</i>	[39–41,71]
			HDR	<i>aCRY, COP1/2, COPS, PHOT, UVR8, VGCC, MAT3, KU80, POLQ</i>	[34]
<i>Nannochloropsis oceanica</i>	Plasmid	Downregulation	None	<i>PEPC1</i>	[38]
<i>Nannochloropsis gaditana</i>	Plasmid	Knockout	Indels	NR; g7988	[49]
<i>Phaeodactylum tricornutum</i>	Plasmid	Disruption	NHEJ based insertion of antibiotic resistant gene cassette	18 different putative transcriptional regulators	[50]
<i>Thalassiosira pseudonana</i>	Plasmid	Knockout	Deletion of a 37 bps fragment in the coding region of the gene by simultaneous targeting of 2 regions on the same gene	<i>URE</i>	[60]
		Disruption	HDR based targeted insertion of antibiotic resistant gene cassette	<i>Silacidin</i>	[60]
<i>Synechocystis sp. PCC 6803</i>	Plasmid	Downregulation	None	<i>phaE, glgC</i> and 4 putative aldehyde reductases/dehydrogenases	[63]
		Knockout	HDR	<i>nblA</i> and <i>isiA</i>	[70]
<i>Synechococcus elongatus</i> PCC 7942	Plasmid	Downregulation	None	<i>glgC, sdhA, and sdhB</i>	[64]
		Knockout	HDR	<i>glgC</i>	[67]
		Knock in	HDR	<i>gltA</i> and <i>ppc</i>	
<i>Synechococcus sp. PCC 7002</i>	Plasmid	Downregulation	None	<i>cpcB, ccmK1, and glnA</i>	[65]
<i>Anabaena sp. PCC 7120</i>	Plasmid	Downregulation	None	<i>glnA</i> and <i>devH</i>	[66]
		Deletion	HDR	<i>nifH</i> and <i>nifD</i>	[70]
<i>Synechococcus elongatus</i> UTEX 2973	Plasmid	Knockout	HDR	<i>nblA</i> and <i>psbA1</i>	[68,70]

RNP, ribonucleoprotein; HDR, homology directed repair.

by SaCas9 was further improved to 16% by recovering the cells after transformation for 1 day at 33 °C and another day at 22 °C before transferring to the antibiotic media plate. The expression of the SaCas9-encoding gene in this experiment was controlled by the HSP70A promoter that typically upregulates the expression of downstream genes after short heat shocks (typically at 40 °C).^[20] Greiner et al.^[19] suggests that the enhanced efficiency in this case could be due to the high Cas9 expression from HSP70A promoter at the elevated recovery temperature (33 °C). Also, SaCas9 derived from the mesophilic host *Staphylococcus aureus* with optimal growth temperature ranging from 37 to 40 °C^[21] might have its ideal activity at higher temperatures, thereby contributing to the improved efficiency. Four photoreceptor genes were successfully targeted by this approach using SaCas9 and, contradictory to SpCas9, intact sequences of the SaCas9-encoding gene were detected from engineered strains, most likely suggesting its reduced toxicity.^[19]

Kao et al. performed CRISPRi and observed decaying mRNA levels of Sp_dCas9 upon sub-culturing of strains up to seven generations.^[22] Linear phenotypic changes were also observed with gradual recovery of the expression of targeted genes in subsequent cultures, suggesting decreased expression of the dCas9 gene in the course of this experiment.^[22] These results differ from the conclusions of Jiang et al., as successful CRISPRi by Kao et al. indicated effective Cas9 expression. In both the studies Cas9 proteins were expressed under same promoter and terminator (Cauliflower Mosaic Virus, CMV-35S). Nevertheless, the Cas9 gene sequences used in the two studies varied up to 14% indicating the effect of codon harmonization on gene expression in *C. reinhardtii*.

As an alternative delivery method, the CRISPR-Cas nuclease proteins purified from (bacterial) production systems, loaded with appropriate guides in vitro, after which the obtained ribonucleoprotein (RNP) complexes can be transformed to the

host of interest. The fact that bacterial production systems are used, circumvent codon optimization for each to-be-edited host. An additional advantage may be that off-target problems are reduced because of a limited half-life of the RNP complex and saturation of the Cas protein with pre-loaded guide RNAs. Indeed, Cas9-RNP delivery to *C. reinhardtii* has substantially resolved issues with plasmid-based Cas9 genome editing.^[23,24] Efficiency of about 1% in generating indels at chromosomal target sites was observed by this approach, even though it varied considerably depending on both the gene targeted and the guide RNA sequence used.^[23–25] Co-transformation of antibiotic resistance genes along with RNP to enhance the selection of transformants resulted in NHEJ-based knock in of antibiotic resistance genes at the target sites.^[23] This opens up opportunities to achieve targeted knock in of gene of interest either by NHEJ or by Homology Directed Repair (HDR). Realizing the limited efficiency of the plasmid-based approach, Greiner et al. also adopted the RNP-based approach for targeting genes which upon disruption could result in non-selectable phenotypes, such as disruption of photoreceptor genes.^[19] To facilitate HDR and achieve errorless DNA repair at an RNP target site, various donor species (plasmids, dsDNA and ssDNA) with 30 bps upstream and downstream homologous flanks were transformed along with the RNP complexes. The linear dsDNA template efficiently integrated to the cleavage site of RNP by NHEJ, whereas the ssDNA template recombined less efficiently but with high chances of flawless HDR. The RNP along with various DNA editing templates were used to disrupt up to eight genes in *C. reinhardtii*, and again the efficiency of achieving each mutants appeared to be gene/guide dependent.^[19] The Cas12a RNP was also used for demonstrating its efficacy in *C. reinhardtii*.^[26] The transformation of Cas12a RNP alone targeting *FKB12* gene depicted targeting efficiency of 0.02% similar to Cas9 RNP. However, remarkable improvement in editing efficiency was observed when ssDNA was co-transformed along with Cas12a RNA for HDR resulting in 29% transformants and 10% errorless HDR mutants.^[26] Apart from *FKB12*, three other genes were also targeted with the same approach for obtaining knockout mutants with an efficiency of 0.5 to 16% and errorless HDR based knockouts with an efficiency of 0.1–10%.^[26]

3. *Nannochloropsis* spp.

Nannochloropsis spp belonging to the class Eustigmatophyceae are considered as model micro-algal strains primarily due to their simple and small genome organization (genome size approximately 30 Mb compared to 120 Mb of *C. reinhardtii*).^[27–29] The industrial relevance for these organisms arise from their potential to accumulate large amounts of tri-acylglycerol (TAG) and poly-unsaturated fatty acids (PUFAs) along with tolerance to wide environmental conditions, such as temperature and light variations.^[29] Vector driven CRISPR-Cas9 based genome editing has been reported in *Nannochloropsis oceanica* and *Nannochloropsis gaditana*.^[30,31] In *N. oceanica*, the frequency of indel mutants generated by Cas9 nuclease targeting the nitrate reductase gene was found to be 1.2% by next generation sequencing. Correspondingly, screening 300 colonies from the transformants resulted in only two confirmed mutants

indicating the very low efficiency of Cas9 in the strains.^[30] Even though reverse transcriptase PCR indicated stable transcripts of Cas9 gene, Western blot analysis could not validate the presence of Cas9 protein indicating the toxicity or feeble expression of Cas9 as observed in *C. reinhardtii*.^[30] In *C. reinhardtii* there were knock in events observed at the Cas9 cleavage site after NHEJ repair, this was not revealed by deep sequencing analysis of *N. oceanica* mutants.^[23,24,30] Instead, up to 0.3% of the sequenced PCR amplicons of the target site after transformation did not align with the wild type target site sequence, which might be random pieces of DNA or the cassette used for transformation inserted at the target site during NHEJ repair. The cytotoxic effects of vector driven expression of Cas9 was not observed in *N. gaditana* cultures, thereby favoring efficient targeted genome editing in the host.^[31] Ajjawi et al. initially developed a *N. gaditana* strain named Ng-Cas9+, that successfully expressed the Cas9 protein from an integrated codon harmonized gene. Co-transformation of an sgRNA guide targeting the gene of interest and a hygromycin resistance cassette to strain Ng-Cas9+ and subsequent selection for growth on hygromycin plates resulted in colonies harboring the hygromycin resistance cassette inserted at the Cas9 cleavage site.^[31] This strategy was used to independently knockdown 18 genes in *N. gaditana* in search for the negative regulators of lipid accumulation. Attenuating the transcription factor *Zn₂Cys₆*, a homolog of fungal *Zn(II)₂Cys₆* DNA binding domain protein using this engineering approach, doubled the lipid production in *N. gaditana*.^[31] Colony PCR screening of transformants that appeared on hygromycin plates revealed that the efficiency of obtaining the 18 mutant genes ranged from 6–78%.^[31]

4. Diatoms

Extending from the equator to regions covered with ice, diatoms thrive in a wide range of marine ecosystems, contributing substantially to global photosynthesis.^[32] Because of their natural capability to accumulate value added compounds along with other environmental and nanotechnology applications, diatoms are considered industrially important microbes.^[32,33] The development of CRISPR-Cas based tools for diatoms were aimed towards developing relatively cost effective and easy genome editing tools for studying fundamental diatom biology via reverse genetics as a basis for obtaining improved strains for biotechnology applications.^[34] To date, CRISPR-Cas systems has been reported to be successfully applied in diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*.^[34,35] In *P. tricornutum*, a codon optimized Cas9 and sgRNA modules were transformed into the host on the same vector without a selectable marker. However, a pAF6 plasmid with zeocin resistance was co-transformed along with Cas9 and sgRNA vector to validate the transformation. Analysis of the transformants by high resonance melting analysis combined with sequencing indicated a mutation frequency of up to 31% for sgRNA targeting the gene *CpSRP54* (chloroplast signal recognition particle 54). Nymark et al. also reports to have achieved a mutation frequency of 25–63% while targeting two other genes in the same species. As diatoms are diploid organism, NHEJ repair of a Cas9-dependent DSB in one allele, may lead to HDR in the other allele, resulting

in identical bi-allelic mutants.^[34] Presence of mixed mutants have been observed in some of the colonies which was hypothesized to be due to the occurrence of cell division prior to initial mutation event.^[34]

Unlike the electroporation transformation used in *Chlamydomonas* and *Nannochloropsis*, biolistic bombardment was used for transformation of diatoms which could fragment the vectors resulting in incomplete introduction of Cas9 gene into the host.^[34–36] Also, co-transformation of selectable and non-selectable vectors has resulted in 40% of the transformants to harbor only the plasmid with selectable marker.^[37] To partially resolve these issues, in *T. pseudonana*, the human codon optimized cas9 gene reported to work in plants^[38] and two sgRNAs targeting 37 nucleotides apart on the urease gene were transformed on a plasmid also harboring an antibiotic resistance marker.^[35] After transformation by micro-particle bombardment, only 12% (4/33) of the obtained colonies harbored an intact cas9 gene; all the colonies in which the cas9 gene was present did have mutations at the target site.^[35] Out of the four colonies (M1, M2, M3 and M4) screened by PCR, the M4 colony was found to be a clean mutant with 37nt deletion between the two target sites. The M2 and M3 colonies showed the presence of both wild type and mutants with 37nt deletion which was confirmed to be mosaic colonies upon screening the sub-clones. Sequence analysis of the M1 colony revealed the presence of a mono-allelic 4 bp deletion at one of the sgRNA cleavage sites.^[35] Overall, the fact that the majority of the mutants were bi-allelic with the designed 37 bp deletion,^[35] indicates efficient Cas9 editing in *T. pseudonana*. The promising Cas9 activity in *T. pseudonana* was applied to achieve efficient HDR and efficiently obtain designed mutants.^[39] Co-transforming the Cas9 and sgRNA on one plasmid with an editing template harboring >500 bp upstream and downstream homologous sequences and an antibiotic resistance marker between the homologous flanks successfully resulted in transformant colonies with 85% HDR efficiency.^[39] The efficient HR along with Cas9 nuclease obtained by this approach is a promising step towards high throughput genome engineering of *T. pseudonana*.

5. Cyanobacteria

The tractability of a prokaryotic organism combined with the photosynthetic capability of eukaryotic microalgae, promises cyanobacteria as a potential cell factory. The efficient conversion of solar energy into biomass, and requirement of low levels of carbon dioxide compared to eukaryotic microalgae result in high growth rates. Moreover, the transformation efficiency, sometimes exploiting natural competence, further elevates the possibilities of evolving selected cyanobacterial strains into a platform organisms for producing biofuels and green chemicals.^[40] The application of CRISPR-Cas9 based genome engineering in cyanobacteria was first reported for *Synechocystis* sp. PCC 6803 where the inactive dCas9 was implemented for target gene downregulation.^[41] Apart from successful single knockdown of genes coding for GFP, polyhydroxyalkanoate synthase and ADP-glucose phosphorylase with varying efficiencies, simultaneous knockdown of up to four genes coding for putative aldehyde dehydrogenases/reductases were also

achieved in this species.^[41] CRISPRi-mediated gene down-regulation has also been reported for *Synechococcus elongatus* PCC 7942 where the expression of an exogenous eYFP gene has been suppressed down to 1% of the control, and the expression of an endogenous gene *glgC* down to 6%.^[42] Two genes encoding subunits of the succinate dehydrogenase (*sdhA* and *sdhB*) were also down-regulated to 19 and 33% compared to control levels resulting in an approximate 12.5% increase in succinate production.^[42] Once the activity of CRISPRi in *Synechococcus* sp. PCC 7002 was confirmed by downregulating the heterologous YFP expression to 0.02% of the control, native genes encoding subunits of the Phycobilisome (*cpbB*) and the Carboxysome (*ccmK1*) were successfully downregulated.^[43] The moderate repression of the glutamine synthetase I (*glnA*) gene by CRISPRi doubled the lactate production in strain PCC 7002, through a series of metabolic processes enhancing the flux of carbon towards pyruvate.^[43] In *Anabaena* sp. PCC 7120, the *glnA* gene was downregulated by 80% using the CRISPRi, resulting in accumulation of ammonium.^[44] The *devH* gene essential for heterocyst development in PCC 7120 was also repressed using CRISPRi, completely abolishing the growth under nitrogen fixing conditions.^[44] The cytotoxic effects of Cas9 protein was observed in strains UTEX 2973 and PCC 7942 of the species *Synechococcus elongatus*.^[45,46] The constitutive expression of the Cas9 protein was found to be toxic in UTEX 2973 even at the minimal levels; this problem was overcome by expressing the Cas9 protein from a vector that cannot replicate at optimal temperature of *Synechococcus*, and thereby facilitating Cas9 expression only during a limited period after transformation. Absence of NHEJ in most prokaryotes in the event of DSB caused by Cas9 is generally lethal, unless a HDR based repair template is provided. Therefore, an upstream and downstream homologous regions of the target gene were incorporated into the plasmid, allowing for homologous recombination-based removal of a target gene, and counter-selecting of wild type cells by Cas9.^[46] This approach resulted in successful knock out of *nblA* gene involved in degradation of photosystem-associated proteins with 100% efficiency.^[46,47] In strain PCC 7942, the activity of Cas9 in inducing DSB resulting in cell death was confirmed upon observing proportional reduction in transformants with increase in the dosage of plasmid harboring Cas9 and sgRNA targeting the host genome for transformation. In line with the observation made in other organisms, the HDR was found to be enhanced in PCC 7942 by the induction of DSB by Cas9.^[6,18,45] This Cas9-assisted HDR was used for metabolic engineering of PCC 7942 by knocking in genes coding for phosphoenolpyruvate carboxylase and citrate synthase to enhance the carbon flux towards oxidative pathway of TCA cycle. Combining these knock-ins with the knock-out of the glucose-1-phosphate adenylyl transferase gene to block the conversion of glucose to glycogen, to increase the carbon flux towards the glycolysis, resulted in an 11-fold increase in succinate titer.^[45]

The toxicity of Cas9 was the bottleneck that restricted wide application of this system in cyanobacteria for genome engineering.^[46] Replacement of Cas9 with Cas12a considerably solved these problems.^[48] Cas12a has been successfully employed for obtaining marker-less mutants of various cyanobacterial species including *Synechococcus elongatus* UTEX

2973, *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120.^[48] Homologous flanks in the upstream and downstream part of the target region (both 1kb) were introduced into the same vector expressing the Cas12a and a crRNA guide was used for HDR based generation of mutants followed by counter selection with Cas12a. Sanger sequencing and PCR of the target site followed by phenotypic characterization confirmed the mutants with knock-ins, knock-outs, and point mutations. In *Synechococcus*, a point mutation in the codon of the *psbA1* gene (S246A) was achieved among 25% of the colonies screened initially; re-streaking the eight colonies thrice in media with antibiotic to maintain the plasmid resulted in 75% of the colonies having the desired genotype.^[48] A similar observation was made during knocking in the YFP gene, where initially 20% of the colonies were segregated mutants and re-streaking the colonies twice on appropriate antibiotic plates yielded 60% mutant colonies. Also, the deletion of *nblA* gene resulted in 90% segregated mutants after three generations of re-streaking.^[48] In *Synechocystis*, the efficiency of obtaining *nblA* gene deletion was comparatively reduced (45%), probably due to the high ploidy level in this species (up to 50 chromosomes per cell), and to the presence of two adjacent copies of the target gene on each chromosome.^[49] Introduction of a point mutation in the *isiA* gene and a gene insertion by replacement of *nblA* gene with YFP was achieved with very high efficiency in *Synechocystis* where 85% of the obtained colonies were segregated mutants.^[48] In *Anabaena*, deletion of first 400 bps of the *nifH* gene yielded the designed mutants in 60% of the colonies. Likewise, point mutation of the *nifD* gene and replacement of *nifH* gene with YFP resulted in 60% segregated mutants.^[48]

6. Conclusions

Genome editing in a wide variety of species has been simplified with efficient and successful application of the CRISPR-Cas technology. However, the cytotoxic effects of the Cas9 nuclease has been a hurdle in exploiting the complete potential of this system in at least some of the microalgal species.^[4,16,19,30,35,45,46] In *Chlamydomonas*, the Cas9 was inferred to be toxic in transformants based on unsuccessful detection of even weak expression of the (intact) Cas9/dCas9-encoding genes.^[4] The molecular basis of this cytotoxic effect of Cas9 has been proposed to be off target cleavage of host genome. Nevertheless, this does not explain observations on inhibitory effects of dCas9, a catalytically inactive Cas9 variant. In another study, however, the successful expression of dCas9 was achieved under the same promoter and terminator for downregulation of various genes.^[22] Whereas the former study used a *Chlamydomonas* codon-optimized dCas9-encoding gene, the latter study used a 15% different *Zea mays* codon-optimized sequence.^[4,22] This observation indicates the importance of factors such as codon harmonization to improve functional protein production. Adding to the proposed Cas9 cytotoxicity, the diminished expression of Cas9 due to yet unknown factors might be responsible for the feeble efficiency of the tool as observed in *C. reinhardtii* and *N. oceanica*. Moreover, the presence of introns and their role in regulating gene expressions in eukaryotes could also be detrimental, e.g., the genome of *Chlamydomonas* contains about

8.5 introns per gene.^[50] Similarly, genomes from micro-algae such as *Nannochloropsis*, *Phaeodactylum* and *Thalassiosira* contain 1.7, 0.8 and 1.5 introns per gene, respectively.^[28,51] The introduction of introns into the coding sequence of some exogenous proteins has been reported to considerably enhance the expression in *Chlamydomonas*^[52]; hence, the introduction of introns could contribute to improved functional Cas9 production. Also, fusing an antibiotic resistance gene to a heterologous gene via a self-cleaving 2A peptide sequence and subsequent selection on antibiotic plates has resulted in transformants with up to 100-fold increased levels of heterologous protein production.^[53] This strategy could be employed for alleviating Cas9 expression and thereby increase the efficiency of generating mutants.

Because the efficiency of implementing plasmid-based Cas9 for genome editing in microalgae was minimal, a breakthrough has been the delivery of RNPs by electroporation.^[19,23,24] However, in cyanobacteria the toxicity problem of Cas9 was solved substantially by replacing it with Cas12a which at least in some cases has resulted in successful genome editing.^[48] The ability of Cas12a to process its own crRNA guides has been exploited for efficient multiplex gene editing in various species.^[12,54] These results indicate the possibilities of expanding Cas12a into the micro-algae for efficient and high-throughput genome engineering. Apart from the SpCas9 and Cas12a other variants of CRISPR systems which could possibly emerge as genome editing tools were reviewed recently.^[55] The improved performance of SaCas9 compared to SpCas9 in *C. reinhardtii*^[19] also indicates the possibilities of applying different unexplored variants of Cas9 and Cas12a to assess their functionality and toxicity effects. The recent characterization of thermostable Cas9 variants may provide a platform for genome engineering of cyanobacterial species adapted to extreme conditions.^[56] It is important to realize that the potential of CRISPR systems is not confined to the widely used Cas9, and that extending engineering studies of model and non-model strains by using distinct Cas nuclease variants could further expand the toolbox for genome engineering of photosynthetic microorganisms and revolutionizing their development as industry-relevant cell factories.

Abbreviations

CRISPR-Cas, Clustered Regularly Interspaced Palindromic Repeats and Associated proteins; CMV, Cauliflower Mosaic Virus; CRISPRi, CRISPR interference; crRNA, CRISPR RNA; dCas9, dead Cas9; GFP, Green Fluorescent Protein; HDR, Homology Directed Repair; NHEJ, Non-Homologous End Joining; nt, nucleotides; PAM, Protospacer Adjacent Motif; PUFA, Poly Unsaturated Fatty Acids; RNP, Ribonucleoprotein; saCas9, *Staphylococcus aureus* Cas9; sgRNA, single guide RNA; spCas9, *Streptococcus pyogenes* Cas9; TAG, Tri-acylglycerol; TALEN, Transcription Activator Like Effector Nuclease; tracrRNA, trans-activating crRNA; YFP, Yellow Fluorescent Protein; ZFN, Zinc Finger Nuclease.

Acknowledgements

This work is supported by the project titled "Microalgae As a Green source from Nutritional Ingredients for Food/Feed and Ingredients for Cosmetics by cost effective New Technologies" [MAGNIFICENT], funded

by Bio-based Industries Joint Technology Initiative under European Commission [project ID: 745754]

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

Cas12a (Cpf1), Cas9, CRISPR-Cas, cyanobacteria, genome editing, microalgae

Received: December 14, 2017

Revised: January 20, 2018

Published online:

- [1] R. H. Wijffels, M. J. Barbosa, M. H. Eppink, *Biofuels, Bioproducts and Biorefining* **2010**, *4*, 287.
- [2] B. Wang, Y. Li, N. Wu, C. Q. Lan, *Appl. Microbiol. Biotechnol.* **2008**, *79*, 707.
- [3] I. Sizova, A. Greiner, M. Awasthi, S. Kateriya, P. Hegemann, *Plant J.* **2013**, *73* 873; F. Daboussi, S. Leduc, A. Maréchal, G. Dubois, V. Guyot, C. Perez-Michaut, A. Amato, A. Falciatore, A. Juillerat, M. Beurdeley, *Nature Commun.* **2014**, *5*, 3831; O. Kilian, C. S. Benemann, K. K. Niyogi, B. Vick, *Proc. Natl. Acad. Sci.* **2011**, *108*, 21265; J. C. Lozano, P. Schatt, H. Botebol, V. Vergé, E. Lesuisse, S. Blain, I. A. Carré, F. Y. Bouget, *Plant J.* **2014**, *78*, 1073.
- [4] W. Jiang, A. J. Brueggeman, K. M. Horken, T. M. Plucinak, D. P. Weeks, *Eukaryotic Cell* **2014**, *13*, 1465.
- [5] E. Charpentier, L. A. Marraffini, *Curr. Opin. Microbiol.* **2014**, *19*, 114; J. A. Doudna, E. Charpentier, *Science* **2014**, *346*, 1258096; P. D. Hsu, E. S. Lander, F. Zhang, *Cell* **2014**, *157*, 1262.
- [6] P. Mali, L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville, G. M. Church, *Science* **2013**, *339*, 823.
- [7] L. S. Qi, M. H. Larson, L. A. Gilbert, J. A. Doudna, J. S. Weissman, A. P. Arkin, W. A. Lim, *Cell* **2013**, *152*, 1173.
- [8] M. H. Larson, L. A. Gilbert, X. Wang, W. A. Lim, J. S. Weissman, L. S. Qi, *Nature Protocols* **2013**, *8*, 2180.
- [9] P. Mohanraju, K. S. Makarova, B. Zetsche, F. Zhang, E. V. Koonin, J. van der Oost, *Science* **2016**, *353*, aad5147.
- [10] A. Endo, M. Masafumi, H. Kaya, S. Toki, *Sci. Rep.* **2016**, *6* 38169; J. K. Hur, K. Kim, K. W. Been, G. Baek, S. Ye, J. W. Hur, S.-M. Ryu, Y. S. Lee, J.-S. Kim, *Nature Biotechnol.* **2016**, *34*, 807; Y. Kim, S.-A. Cheong, J. G. Lee, S.-W. Lee, M. S. Lee, I.-J. Baek, Y. H. Sung, *Nature Biotechnol.* **2016**, *34*, 808; D. Kim, J. Kim, J. K. Hur, K. W. Been, S.-h. Yoon, J.-S. Kim, *Nature Biotechnol.* **2016**; B. P. Kleinstiver, S. Q. Tsai, M. S. Prew, N. T. Nguyen, M. M. Welch, J. M. Lopez, Z. R. McCaw, M. J. Aryee, J. K. Joung, *Nature Biotechnol.* **2016**, *34*, 869; E. Tóth, N. Weinhardt, P. Bencsura, K. Huszár, P. I. Kulcsár, A. Tálás, E. Fodor, E. Welker, *Biology Direct* **2016**, *11*, 46; R. Xu, R. Qin, H. Li, D. Li, L. Li, P. Wei, J. Yang, *Plant Biotechnol. J.* **2017**, *15*, 713; R. Verwaal, N. Buiting-Wiessenhaan, S. Dalhuijsen, J. A. Roubos, *Yeast* **2017**.
- [11] B. Zetsche, J. S. Gootenberg, O. O. Abudayyeh, I. M. Slaymaker, K. S. Makarova, P. Essletzbichler, S. E. Volz, J. Joung, J. van der Oost, A. Regev, *Cell* **2015**, *163*, 759.
- [12] B. Zetsche, M. Heidenreich, P. Mohanraju, I. Fedorova, J. Kneppers, E. M. DeGennaro, N. Winblad, S. R. Choudhury, O. O. Abudayyeh, J. S. Gootenberg, *Nature Biotechnol.* **2017**, *35*, 31.
- [13] I. Fonfara, H. Richter, M. Bratovič, A. Le Rhun, E. Charpentier, *Nature* **2016**, *532*, 517.
- [14] M. A. Moreno-Mateos, J. P. Fernandez, R. Rouet, C. E. Vejnar, M. A. Lane, E. Mis, M. K. Khokha, J. A. Doudna, A. J. Giraldez, *Nature Commun.* **2017**, *8*, 2024.
- [15] E. H. Harris, *Ann. Rev. Plant Biol.* **2001**, *52*, 363.
- [16] W. Z. Jiang, D. P. Weeks, *Algal Res.* **2017**, *52*, 474.
- [17] K. L. Kindle, R. A. Schnell, E. Fernández, P. A. Lefebvre, *J. Cell Biol* **1989**, *109*, 2589.
- [18] W. Jiang, D. Bikard, D. Cox, F. Zhang, L.A. Marraffini, *Nature Biotechnol.* **2013**; 233; H. L. Li, N. Fujimoto, N. Sasakawa, S. Shirai, T. Ohkame, T. Sakuma, M. Tanaka, N. Amano, A. Watanabe, H. Sakurai, *Stem Cell Reports* **2015**, *4*, 143; M. E. Pyne, M. Moo-Young, D. A. Chung, C. P. Chou, *Appl. Env. Microbiol.* **2015**, *81*, 5103; S. Turan, C. Zehe, J. Kuehle, J. Qiao, J. Bode, *Gene* **2013**, *515*, 1.
- [19] A. Greiner, S. Kelterborn, H. Evers, G. Kreimer, I. Sizova, P. Hegemann, *Plant Cell* **2017**, tpc. 00659.2017.
- [20] M. Schroda, D. Blöcker, C. F. Beck, *Plant J.* **2000**, *21*, 121.
- [21] M. I. Halpin-Dohnalek, E. H. Marth, *J. Food Protection* **1989**, *52*, 267; P. Zangerl, H. Asperger, *Progress Indus. Microbiol.* **2003**, *37*, 91.
- [22] P.-H. Kao, I.-S. Ng, *Bioresource Technol.* **2017**, *245*, 1527.
- [23] S.-E. Shin, J.-M. Lim, H. G. Koh, E. K. Kim, N. K. Kang, S. Jeon, S. Kwon, W.-S. Shin, B. Lee, K. Hwangbo, *Scientific Rep.* **2016**, *6*, 27810.
- [24] K. Baek, D. H. Kim, J. Jeong, S. J. Sim, A. Melis, J.-S. Kim, E. Jin, S. Bae, *Scientific Rep.* **2016**, *6*, 30620.
- [25] K. Baek, J. Yu, J. Jeong, S. J. Sim, S. Bae, E. Jin, *Biotechnol. Bioeng.* **2017**, *115*, 719.
- [26] A. Ferenczi, D. E. Pyott, A. Xipnitou, A. Molnar, *Proc. Nat. Acad. Sci.* **2017**, *114*, 13567.
- [27] K. Pan, J. Qin, S. Li, W. Dai, B. Zhu, Y. Jin, W. Yu, G. Yang, D. Li, *J. Phycol.* **2011**; 1425; R. Radakovits, R. E. Jinkerson, S. I. Fuerstenberg, H. Tae, R. E. Settlege, J. L. Boore, M. C. Posewitz, *Nature Commun.* **2012**, *3*, 686; A. Vieler, G. Wu, C.-H. Tsai, B. Bullard, A. J. Cornish, C. Harvey, I.-B. Reca, C. Thornburg, R. Achawanantakun, C. J. Buehl, *PLoS Genetics* **2012**, *8*, e1003064; L. Wei, X. Huang, Z. Huang, Z. Zhou, *Bioresource Technol.* **2013**, *147*, 534.
- [28] E. C. Carpinelli, A. Telatin, N. Vitulo, C. Forcato, M. D'Angelo, R. Schiavon, A. Vezzi, G. M. Giacometti, T. Morosinotto, G. Valle, *Mol. Plant* **2014**, *7*, 323.
- [29] D. Wang, K. Ning, J. Li, J. Hu, D. Han, H. Wang, X. Zeng, X. Jing, Q. Zhou, X. Su, *PLoS Genetics* **2014**, *10*, e1004094.
- [30] Q. Wang, Y. Lu, Y. Xin, L. Wei, S. Huang, J. Xu, *Plant J.* **2016**, *88*, 1071.
- [31] I. Ajjawi, J. Verruto, M. Aquil, L. B. Soriaga, J. Coppersmith, K. Kwok, L. Peach, E. Orchard, R. Kalb, W. Xu, *Nature Biotechnol.* **2017**, *35*, 647.
- [32] A. Bozarth, U.-G. Maier, S. Zauner, *Appl. Microbiol. Biotechnol.* **2009**, *82*, 195.
- [33] T. Lebeau, J.-M. Robert, *Appl. Microbiol. Biotechnol.* **2003**, *60*, 612.
- [34] M. Nymark, A. K. Sharma, T. Sparstad, A. M. Bones, P. Winge, *Scientific Rep.* **2016**, *6*, 24951.
- [35] A. Hopes, V. Nekrasov, S. Kamoun, T. Mock, *Plant Methods* **2016**, *12*, 49.
- [36] T. B. Jacobs, P. R. LaFayette, R. J. Schmitz, W. A. Parrott, *BMC Biotechnol.* **2015**, *15*, 16.
- [37] A. Falciatore, R. Casotti, C. Leblanc, C. Abrescia, C. Bowler, *Marine Biotechnol.* **1999**, *1*, 239.
- [38] V. Nekrasov, B. Staskawicz, D. Weigel, J.D. Jones, S. Kamoun, *Nature Biotechnol.* **2013**; 691; K. Belhaj, A. Chaparro-Garcia, S. Kamoun, V. Nekrasov, *Plant Methods* **2013**, *9*, 39; C. Brooks, V. Nekrasov, Z. B. Lippman, J. Van Eck, *Plant Physiol.* **2014**, *166*, 1292.
- [39] N. Belshaw, I. Grouneva, L. Aram, A. Gal, A. Hopes, T. Mock, *bioRxiv* **2017**, 215582. <https://doi.org/10.1101/215582>
- [40] J. Yu, M. Liberton, P. F. Cliften, R. D. Head, J. M. Jacobs, R. D. Smith, D. W. Koppenaal, J. J. Brand, H. B. Pakrasi, *Scientific Rep.* **2015**, *5*; A.

- Parmar, N. K. Singh, A. Pandey, E. Gnansounou, D. Madamwar, *Bioresource Technol.* **2011**, *102*, 10163.
- [41] L. Yao, I. Cengic, J. Anfelt, E. P. Hudson, *ACS Synthetic Biol.* **2015**, *5*, 207.
- [42] C.-H. Huang, C. R. Shen, H. Li, L.-Y. Sung, M.-Y. Wu, Y.-C. Hu, *Microb. Cell Factories* **2016**, *15*, 196.
- [43] G. C. Gordon, T. C. Korosh, J. C. Cameron, A. L. Markley, M. B. Begemann, B. F. Pfeleger, *Metabolic Eng.* **2016**, *38*, 170.
- [44] A. Higo, A. Isu, Y. Fukaya, S. Ehira, T. Hisabori, *Plant Cell Physiol.* **2017**, *59*, 119.
- [45] H. Li, C. R. Shen, C.-H. Huang, L.-Y. Sung, M.-Y. Wu, Y.-C. Hu, *Metabolic Eng.* **2016**, *38*, 293.
- [46] K. E. Wendt, J. Ungerer, R. E. Cobb, H. Zhao, H. B. Pakrasi, *Microb. Cell Factories* **2016**, *15*, 115.
- [47] J. L. Collier, A. Grossman, *EMBO J.* **1994**, *13*, 1039.
- [48] J. Ungerer, H. B. Pakrasi, *Scientific Rep.* **2016**, *6*, 39681.
- [49] K. Zerulla, K. Ludt, J. Soppa, *Microbiology* **2016**, *162*, 730.
- [50] M. Jąkałski, K. Takeshita, M. Deblieck, K. O. Koyanagi, I. Makałowska, H. Watanabe, W. Makałowski, *Biol. Direct* **2016**, *11*, 35.
- [51] S. W. Roy, D. Penny, *Mol. Biol. Evolution* **2007**, *24*, 1447; C. Bowler, A. E. Allen, J. H. Badger, J. Grimwood, K. Jabbari, A. Kuo, U. Maheswari, C. Martens, F. Maumus, R. P. Otilar, *Nature* **2008**, *456*, 239.
- [52] V. Lumbreras, D. R. Stevens, S. Purton, *Plant J.* **1998**, *14*, 441.
- [53] B. A. Rasala, P. A. Lee, Z. Shen, S. P. Briggs, M. Mendez, S. P. Mayfield, *PLoS ONE* **2012**, *7*, e43349.
- [54] M. Wang, Y. Mao, Y. Lu, X. Tao, J.-K. Zhu, *Mol. Plant* **2017**, *10*, 1011.
- [55] J. Murovec, Ž. Pirc, B. Yang, *Plant Biotechnol. J.* **2017**, *15*, 917; S. Nakade, T. Yamamoto, T. Sakuma, *Bioengineered* **2017**, *8*, 265.
- [56] L. B. Harrington, D. Paez-Espino, B. T. Staahl, J. S. Chen, E. Ma, N. C. Kyrpides, J. A. Doudna, *Nature Commun.* **2017**, *8*, 1424; I. Mougiakos, P. Mohanraju, E. F. Bosma, V. Vrouwe, M. F. Bou, M. I. Naduthodi, A. Gussak, R. B. Brinkman, R. Kranenburg, J. Oost, *Nature Communications* **2017**, *8*, 1647.