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НАУКИ О ЗЕМЛЕ

Insight into CRISPR system in Eukaryotic microalgae, a review

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ABSTRACT

Microalgae are more than ever of paramount importance: nutrition, cosmetic, energy, biotechnology tools … Wisely used CRISPR/Cas9 technology can help industries to overcome some issues and increase production yield of some valuable compounds. However, the following review shows that it is too early for both routine and industrial applications. Different transfections techniques were applied, nevertheless, inside the same laboratory different electroporation devices and conditions have been used. Moreover, genomic editing efficiency were often too low. In order to define which technique is the best suited for each microalgae it is urgent to lead some studies comparing the CRISPR mediated genome editing upon different transfection techniques and conditions. This review highlights that the scientific community needs to set for each microalgae the right protocol to follow and be able to compare the results from one laboratory to another. Finally, some techniques were not tested in order to introduce the CRISPR technology inside eukaryotic microalgae and increase the genome-editing yield. In that sense recombinant Cas9 or CPF1 coupled to cell penetrating peptides or gold nanoparticles as well as tunable expression vectors (optogenetic, chemically induced etc…) could be very interesting to develop.

Keys words: Microalgae; cell; peptide; improvement; efficiency; genome; CPF1; Cas9; CRISPR

АННОТАЦИЯ

Микроводоросли имеют как никогда первостепенное значение: инструменты для питания, косметики, энергии, биотехнологии ... Мудро использованная технология CRISPR / Cas9 может помочь промышленности преодолеть некоторые проблемы и увеличить выход некоторых ценных соединений. Тем не менее, следующий обзор показывает, что это слишком рано для обычных и промышленных приложений. Различные методы трансфекции были применены, тем не менее, внутри одной и той же лаборатории были использованы различные устройства электропорации и условия. Более того, эффективность геномного редактирования часто была слишком низкой. Чтобы определить, какой метод лучше всего подходит для каждой микроводоросли, необходимо срочно провести некоторые исследования, сравнивающие CRISPR-опосредованное редактирование генома при различных методах трансфекции и условиях. В этом обзоре подчеркивается, что научное сообщество должно установить для каждого микроводоросля правильный протокол, которым нужно следовать, и иметь возможность сравнивать результаты из одной лаборатории в другую. Наконец, некоторые методы не были протестированы для того, чтобы внедрить технологию CRISPR внутри эукариотических микроводорослей и увеличить выход для редактирования генома. В этом смысле рекомбинантный Cas9 или CPF1, связанный с проникающими в клетку пептидами или наночастицами золота, а также с перестраиваемыми векторами экспрессии (оптогенетическими, химически индуцированными и т. Д.) Может быть очень интересным для разработки.

Ключевые слова: микроводоросли; клетка; пептид; улучшение; эффективность; геном; CPF1; cas9; CRISPR

1. Introduction:

Microalgae are important biological resources that have a wide range of biotechnological and industrial

applications such as; biofuel, bio–sequestration of CO_2, aquaculture, bioremediation, agriculture, cosmetics and recombinant proteins production [1, 2, 3, 4, 5, 6, 7, 8]. Genomic engineering improvement is critical in order to increase further the microalgae production of high–value products and bio–energy [9], while creating organism less demanding and/or more resistant for industry purposes.

Nowadays, although scientist community manage to transform microalgae chloroplast with some successes in order to express proteins of interest, nuclear transformation remains quite difficult, random and labor intensive.

As discussed and reviewed by [10], transformation techniques were applied few microalgae compared to the huge diversity this denomination encompass [11], most of them conducted on *C. reinhardtii* as a model organism. Research teams often use plasmids to conduct their experiments [10], homologous recombination can be used in order to insert transgenes during chloroplast transformation but nuclear transformation remains more a random event [12]. Moreover, stability of the transgene and gene silencing [13, 14] could also occur rendering such transformation process difficult to achieve or almost impossible in few cases. It was pointed out that the homologous recombination frequency in microalgae is, we quote, "too low for adaptation as a recommended technology" [10]. Some exceptions like *Nannochloropsis* sp. strain W2J3B present an efficient homologous recombination process [15] but it was not applicable in other *Nannochloropsis* strains. Indeed, ideally both the industry and the scientists want a technique not only able to knockout genes but also to modify and tune the genome at will, in a precise manner. Traditional techniques do not provide enough precision to do it. Doing a point mutation at a precise location to modify an endogenous enzyme in a clean, controlled, manner is something we could not do easily until recently. Genomic positional effects, random insertion and genomic rearrangements are all limitations that compel researchers spending a lot of time screening for "the right transformant".

The use of plasmids are convenient but if such problems occur looking for an enzyme able to edit and regulate the genome in a "DNA free" fashion could be something valuable if it can enter the cell, being easily directed to the organelle and location of interest.

As a consequence alternatives, were tested to edit the genome using endonucleases. Transcription Activator–Like Effector Nuclease (TALEN) have been in microalgae but results using those two labor intensive techniques seem to be difficult to achieve [15,16,17,18] or Zinc–Finger Nuclease (ZFN) [19] have been used in microalgae but results using those two labor intensive techniques seem to be difficult to achieve.

Clustered regularly interspaced short palindromic repeats (CRISPR) system using the RNA–guided engineered nuclease (RGEN) Cas9 is able to target a specific genomic region thanks to single guide RNA abbreviated sgRNA [20, 21, 22], it that emerged as an easier, versatile and reliable technique to insert, remove and tune the genome. It is a powerful tool for everyone willing to edit the nuclear genome knocking–out or knocking–in genes by Homology Directed Repair (HDR) [20, 23], but also repressing or activating endogenous genes transcription by CRISPR interference [24, 25, 26, 27, 28, 29, 30]. CRISPR/Cas9 technology could circumvent the use of exogenous genes or other DNA sequences thus being more ethically acceptable [31, 32]. It was extensively studied in vegetables like *Arabidopsis thaliana*, tobacco, tomato, maize and rice [33, 34, 35, 36, 37, 38, 39, 40, 41, 42]. Compared to CRISPR/Cas9, TALEN and ZFN are more expensive and time consuming as well [43. Finally, a DNA free CRISPR/Cas9 mediated genomic edition can lead to a "non–GMO" plant or microalgae [31].

Consequently, some studies have been done [44, 45, 46, 47, 48], trying to apply the CRISPR/Cas9 technology to microalgae genetic tailoring, some of them using a preassembled endonuclease. This review aims to provide solutions and future prospects in order to improve the CRISPR system efficiency in microalgae. First and foremost, we present what results have been obtained so far for each microalgae, related to technical context and pointing some issues that deserve to be clarified. After this overview, the discussion will provide guidelines and ideas for future project in order to improve the efficiency in microalgae.

2. Eukaryotic microalgae

2.1. Chlamydomonas reinhardtii

Some studies have been done concerning the use of the CRISPR/Cas9 system in *C. reinhardtii* [44, 47, 50]. Published in 2014, the first attempt used a codon optimized expression vector [45] in *Chlamydomonas* CC–503 strain, a mutant strain lacking an intact cell wall. The authors never used the same strain and nothing was written in a view to informing those decisions. CC–4349cm15mt– [44] and CC–124 [47] were used in further studies. They construct several vectors encoding for both the Cas9 gene and its corresponding sgRNA in conjunction with mutated exogenous reporter genes such as hygromycin, green fluorescent protein, or *Gaussia luciferase*.

In order to assess the ability to mutate the endogenous genome, another strategy targeting the

endogenous FKBP12 gene was set to generate rapamycin resistant *Chlamydomonas* strain [51, 31] assessing the capability to mutate the *C. reinhardtii* genome. Information is gathered about the construct and techniques are herein presented in table 1. Like, hygromycin, GFP, *Gaussian luciferase* genes, the FKB12 targeted sequence is in close proximity or contain restriction enzyme sites. The goal was to digest none mutated DNA thus enriching the PCR pool with mutated sequences. This technique is based on several suppositions and only reveals mutants with a destroyed restriction site; then it only shows a proportion of all mutagenic events. Nevertheless, the authors questioned themselves about the efficacy of CRISPR/Cas9 in microalgae saying that after 16 independent [53].

They discover that the Cas9 by itself is toxic in *Chlamydomonas* using a catalytically dead Cas9 (D10A/H840A) double mutant called dCas9. It is the same dCas9 used in CRISPR interference (CRISPRi) in order to modulate transcription as described in many articles [28, 30]. They used antibiotic resistance containing vector in order to enrich the microalgae population with cells harboring the dCas9 encoding construct. After such selection only 6 clones, among 33 picked–up, displayed "intact dCas9" genes but no signal could have been detected by western–blot analysis. Of course increasing efficacy of CRISPR/Cas9 technology in microalgae will be as important as controlling its toxicity. Cas9 mRNA was detectable but not the corresponding ribonucleoprotein. This also gives rise to concern about CRISPR interference (CRISPRi) in *C. reinhardtii* since this technique would require an intact dCas9 to modulate the genome. At this point, what causes Cas9 protein down regulation or degradation remains unraveled. Although, we can notice that toxicity is probably not completely dependent on DNA processing since catalytically dead Cas9 seems to be toxic as well.

A first part of the answer was investigated in 2016 [46, 49]. We will describe their methods and findings before pointing out details each one should have in mind in the future. Given the alleged Cas9 toxicity in *C. reinhardtii* the research group opted for a "preassembled" ribonucleoprotein (RNP). It was already used before in several organism including plants [54, 55, 56, 57, 58, 59, 60, 61, 62]. Use of DNA–free RNP Cas9 is considerably faster compared to plasmid driven Cas9–sgRNA production, considering all the steps the tailoring and testing of a plasmid with optimized codon and ribosome binding sequence (RBS) can take. But more than that, following the cell entrance and subsequent DNA cut, preassembled Cas9 seems to be short lived, potentially reducing off–target and mosaicism (Kim et al., 2014; Ramakrishna et al., 2014).

Shin et al. [49] targeted three distinct genes: MAA7, CpSRP43 and ChlM by electroporating the ribonucleoprotein with or without resistance encoding vector into CC–124 *C. reinhardtii* cells. In order to test the efficacy of each sgRNA they tested it using Cas9 digestion of PCR amplified genomic regions encompassing the Cas9 cut site. The sgRNA unable to promote a proper DNA cleavage during the in–vitro assay was unable to generate any mutant, suggesting that a preliminary in–vitro test for each sgRNA is a wise move we should keep in mind for further studies before running time consuming mutagenic screen, the authors recommended it rightly, then it is not surprising giving past publications [55, 56]. As we can see later the other papers using CRISPR in microalgae never tested their sgRNA in comparable in–vitro test.

MAA7 mutant is 5–fluoroindole resistant, the wild type version encoding the tryptophan synthase beta subunit (TSB) [62, 63, 64], while CpSRP43 and ChlM mutants can be selected by a bleached color phenotype. Both CpSRP43 and ChlM were co–transfected with a hygromycin resistance–encoding vector in order to select the transfected population.

The total targeting efficacy per cell of MAA7 reached 8.9×10^{-8} and a total of 8 clones were isolated. Seven of them displayed 3bp long indels leading to point mutation instead of the frameshift they expected in order to generate a proper knockout, the eighth clone sequencing showing a 33 bp insertion. Conveniently, the mutation modified a conserved RPDAN [46, 52, 48, 31, 49] amino acid motif critical for the Alpha–Beta subunit interaction which is necessary for the enzymatic reaction [63, 64].

Previous work reported the use of NHEJ–repair mediated knock–in for both CpSRP43 and ChlM gene modification. The three isolated mutants for the CpSRP43 gene were light green similarly to CC–4561 and CC–4562 deletion mutants used as positive control. Unfortunately, PCR and sequencing analysis revealed a major problem. 2650 bp, from the selection vector, were integrated at the Cas9 cut site together with a 13 bp sequence with unknown origin. It was a single copy insertion. The selection vector called Vec–2 do not contain any sequence matching the cutting site region thus, they concluded that the insertion was NHEJ–repair mediated [66]. An interesting fact not discussed in that paper appeared in figure 5. PCR analysis show that several "Regions" of the vector are indeed present in the mutants and not in the wild-type. The control of the

experiment is the vector alone, in absence of a proper dCas9 that should have been used. The figure 5, part b [49] shows clearly that both "Region B" (1662 bp) and the hygromycin resistance region (287 bp) of the vector were inserted in the Vec–2 condition, meaning without the Cas9. The clone CpSRP43 20–1, electroporated with both Vec–2 and Cas9–sgRNA complex, also display a similar pattern and colony with a dark–green color probably coming from a cell where only the Vec–2 entered. The emerging question is, why "Region B" and hygromycin resistance region integrate by them self into the genome and not parts of "Region C" or "Region A"? Is the electroporation the suitable transformation approach in this case? Is there any other technics that could lead to a different result? To answer those questions we need to compare various transformation technique and strategies, which still has to be explored.

The ChlM case confirmed that something is going out of control. The main purpose using the RNA–guided engineered nucleases (RGEN) CRISPR/Cas9 being to do a precise and controlled modification of the genome, unwanted genomic integrations of exogenous DNA is thus inconvenient. Indeed, among 10 mutants displaying a lighter color phenotype, most of them contain at least one copy of the vector inserted, some containing two copies of it. Moreover, they described several rearrangement of the vector sequence together with some indels inserted next to the CRISPR/Cas9 cutting site as seen previously. Again, no homology was found between the vector and the ChlM gene sequence targeted by the Cas9 enzyme, leading to the conclusion that NHEJ–repair mediate those insertions. Interestingly, "Figure7 part a" [49], showing the result of the PCR analysis for several region of the vector do not show a control using the vector alone but only a regular wild–type. "Part b" of the same figure present the maps of the rearrangements for each mutants, which are apparently random, the author could not explain those genomic events. As a consequence it is difficult to draw predictions for further studies. Shin et al. [49] methods improved the CRISPR/Cas9 efficacy in *Chlamydomonas* compared to previous results obtained by [47]. The MAA7–2, CpSRP43 and ChlM experiments have a total targeting efficiency (per cell) of 8.9×10^{-8}, 3.3×10^{-8} and 5.0×10^{-8} respectively. The math being done using the number of cells used for electroporation as a total. Off–targets research do not find any mutations for MAA7 and CpSRP43 experiments, nevertheless both ChlM–4 and ChlM–21 clones are knock–in with insertions at another location.

Baek et al. [46] used the DNA–free Cas9 RNA–guided engineered nuclease to modify the CpFTSY and ZEP genes electroporating the RNA guided Cas9 complex into CC–4349cm15mt– strain cells. Reading the title we can think about a simultaneous double gene knockout but it is a two–step double gene knockout. This third *Chlamydomonas* paper [46] do not bring new technical insights compared to the Shin SE et al. paper [49] but it confirmed the results obtained using DNA–free RGENs. They generate at first a ZEP knockout called ΔZEP and then modified the CpFTSY gene obtaining a ΔZEP/ΔCpFTSY transformant.

ZEP gene encodes for Zeaxanthin epoxidase implicated in antheraxanthin and violaxanthin synthesis, CpFTSY is a receptor for chloroplast signal recognition particle (CpSRP) genes [67]. ZEP knock–out will enable Zeaxanthin accumulation and the additional CpFTSY knock–out is aimed to reduce chlorophyll antenna size allowing a higher intensity for the saturation of photosynthesis and a greater maximum photosynthetic rates (Pmax) [67, 68, 69] improving mass culture productivity under hight light. Indeed, the ΔZEP/ΔCpFTSY mutant displayed a greater growth and a Pmax 54 % higher at maximum light intensity compared to the ΔZEP mutant. Three sgRNAs directed against the CpFTSY gene were tested producing 0.007 %, 0.12 % and 0.272 % indel frequency.

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Table 1: Summary of processing techniques used in CRISPR system in Eukaryotic microalgae studies.

Transformation technique	Electroporation				Biolistic
Transformation protocol and condition	Bio–Rad Gene Pulser II. Cells were resuspended TAP 60mM sucrose to a density of $4x10^8$ cells/ml. 250 µL of cells was mixed with 2 g plasmids in an electroporation cuvette (4 mm gap) chilled for 5 min in a 16 °C water bath prior to electroporation. One pulse at 0.75 kV and 25 µF without resistance was applied.	Biorad CM 830 Square Wave Electroporation System using cuvettes (2 mm gap). Cas9 (10 to 40µg) and sgRNAs (7.5 to 30 µg) were incubated together at 37 °C for 30 min, and 300 µL (quantity must be in number cells not as a volume) were placed in the cuvette and cooled on ice for 5 min. Electroporation was done at 250 V and 15 ms interval.	Bio–Rad Gene Pulser Xcell™ Electroporation Systems. 2 µg of linearized plasmid. 200 µg of Cas9 protein and 140 µg of sgRNA in a 4 mm gap electroporation cuvette during 5 min at room temperature. $5x10^5$ cells were electroporated at 600 V and 50 µF. Cf. GeneArt® Chlamydomonas Engineering Kits.	Cells was mixed with 2 µg vector in a 2 mm gap electroporation cuvette. BTX ECM 630 electroporation device was used with 11 kV/cm field strength, 50µF capacitance, and 600 Ohm shunt resistance.	Bio–Rad Biolistic PDS 1000/He Particle Delivery System. Tungsten M17 microcarriers (Bio–Rad) were coated with 2.5 µg of vector. $0.5x10^8$ cells was plated and grown for 1 day.
Microalgae and strain	*Chlamydomonas reinhardtii*, strain CC–503 (lacking intact cell wall)	*Chlamydomonas reinhardtii*, strain CC–124	*Chlamydomonas reinhardtii*, strain CC–4349 cw15 mt–	*Nannochloropsis oceanica*, strain IMET1	*Phaedactylum tricornutum* cells, strain CCMP2561
Cas9 version	Cas9 encoding vector	DNA–free RNA–guided engineered nuclease (RGENs)	DNA–free RNA–guided engineered nuclease (RGENs)	Cas9 encoding vector	Cas9 encoding vector
Constructs	Cas9 driven by Cauliflower mosaic virus (CaMV) 35S promoter and terminated by nopaline synthetase gene termination region (Tnos). sgRNA gene was flanked by *Arabidopsis thaliana* U6 promoter and terminator.	None; the synthetised ribonucleoprotein is electroporated directly with or without an antibiotic resistance encoding vector.	None; the synthetised ribonucleoprotein is electroporated directly with or without an antibiotic resistance encoding vector.	Vector harboring a hygromycin resistance (HygR). A codon optimized Cas9 controled by violaxanthin/chlorophyll (a) binding protein promoter (Pvcp) and α–tublin termination (Tatub) region. The sgRNA was driven by a V–type ATPase promoter (Patpase) and terminated with ferredoxin terminator (Tfd).	Cas9 controled by the *P. tricornutum* LHCF2 promoter and LHCF1 terminator sequence. sgRNA expression was controled via *P. tricornutum* U6 snRNA promoter and poly–T termination signal from Chr8 (CM000611.1).

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Endogenous gene targeted	FKB12 gene (XM001693563.1; phytozome Cre13.g586300.t1.2)	MAA7 gene (XM_001703345 and XP_001703397); CpSRP43 gene (XM_001703652 and XP_001703704)); ChlM gene (XM_001702328 and XP_001702380).	CpFTSY and ZEP genes (the author do not mension the exact NCBI map they use)	Nitrate Reductase NR (NR; g7988)	CpSRP54 gene (Draft ID: Phatr2_35185, Chr7: NC_011675.1)
sgRNA	FKBP12 sgRNA 5–GGCGTGGCCCAGATGTCCAA–3	MAA7–2 sgRNA 5–CAUAGCGACCAUUUGCGUCC–3; CpSRP43 sgRNA 5–CGAUUCCGGCCUGCACCGGC–3; ChlM sgRNA 5–CCCGCCCGGCUGUGGCCCGG–3	CpFTSY sgRNA 5–CGATCTTCAGAGCAGTGCGG–3; ZEP sgRNA 5–TCCGGCGAACGCACCTGGAT–3	NR sgRNA 5–CAGAGCAAGGGCTTCAGCTG–3	CpSRP54 sgRNA 5–CCGCCCTTCGTGAAGTACGT–3
Number of cell used	$1,6*10^{9}$ (16 transformations were done and pulled together)	$0.9x10^{8}$	$5x10^{5}$	n/a	$0.5x10^{8}$
Efficiency claimed	$1,6x10^{-9}$	from $3.3x10^{(-8)}$ to $8.9x10^{(-8)}$	0.56 % for ΔCpFTSY; 0.45 % for ΔZEP and 1,1% for ΔZEP/ΔCpFTSY	1.22 % for flask no 5. 0.122 % for all ten flasks.	31 % (or $16x10^{-8}$)
Comments	Dead–Cas9 experiment have shown that Cas9 is toxic per se.	Small Indels inducing point mutations of the MAA7 encoding protein. Several plasmid DNA insertions at the Cas9 cut site,	The same did both articles using DNA–free RGENs, yet they use different protocols and even different Chlamydomonas strain without justifying why.	They worked on one flask out of ten (flask nº 5). Then the results do not represent the total efficacy but the results for the flask nº5 only.	8 out of 23 transformant obtained after culture in selection media. Single nucleotide insertion, small deletion and vector derived DNA integration. If we use the initial number of cells, we have an efficiency of 8 cells out of $0.5x10^{8}$, thus $16x10^{-8}$.
References	[52]	[49]	[46]	[50]	[48]

The best one produced 0.56 % mutation frequency in a second round of experiment. Five ZEP sgRNA were evaluated by targeted deep sequencing as well, indel frequency spreading from 0.094 % up to 0.456 %. Of course, the best sgRNAs results were encouraging, yet the efficacy varies a lot from one sgRNA to another. Best results were encouraging and thus emphasized. Highlighting the best results obtained is of course something that should be done but looking at the whole data we see results varying from one sgRNA to another but also for the same sgRNA: in the CpFTSY example the efficacy of the sgRNA double at the second use without any explanation. The zeaxanthin example is a great one because it shows what CRISPR mediated mutation could be very important in order to generate cell lines with improved high–value compound production. While the wild type Zeaxanthin quantity was 0.0062 ± 0.0005 fmol per cell, both ΔZEP and ΔZEP/ΔCpFTSY show amazing increase, up to 0.1376 ± 0.0007 (22 fold increase) and 0.0827 ± 0.0009 (13 fold increase) respectively.

2.2. Phaedactylum tricornutum

As reported in the introduction [18] and [17] managed to edit *Phaedactylum tricornutum* CCMP2561 strain genome using transcription activator–like–effector nucleases (TALEN). Nymark et al. [48] used Biolistic mediated co–transformation of a home maid pKSdiaCas9_sgRNA vector and pAF6 Zeocin resistance vector. Biolistic is DNA–coated gold or tungsten micro–particles delivered at high–velocity such technique was used in various microalgae including *P. tricornutum* [70, 71, 72]. They decided to target a gene implicated in the chloroplast signal recognition particle pathway named CpSRP54. As CpSRP43, those genes are convenient to target because they allow researchers to discriminate mutated cells easily using color alteration phenotype.

The efficacy in this paper appear amazingly high compared to *C. reinhardtii*, giving 31% mutation frequency (8 out of 26 transformants) for CpSRP54 Researchers claimed as well the successful transformation and mutation of two additional genes with mutation frequency ranging from 25 to 63 %, without showing the data. This seems to be a huge improvement compared to *Chlamydomonas* papers previously presented and discussed.

Jiang et al. [52] showed that Cas9 protein is toxic and impossible to detect in their study, in *Phaedactylum tricornutum*, [48] qRT–PCR was used to detect Cas9 levels but never show or talk about protein levels. They assume that Cas9 is not toxic in *Phaedactylum tricornutum* because relatively high levels of both Cas9 mRNA and sgRNA were found. Jiang et al. [52] also had good mRNA levels but they showed that Cas9 protein was absent, perhaps being rapidly degraded inside the cell. Here we cannot rule out that the Cas9 protein production is indeed repressed or the protein degraded so fast that the only time window available is the adaptation just after the transfection event. Scientist should find a same way to evaluate and compare their protocols. Talking about technique efficacy, [47] looked at the number of cell they initially used ($1.6x10^9$ cells) and the number of mutant they obtained, one.

Among eight transformants successfully modified, one presents a 212–bp insertion corresponding to the used vector. As the author reported, such kind of phenomenon occurred in TALEN study [18] using biolistic transformation; although it seems that the integration rate was lower with biolistic compared to the various insertion seen in *Chlamydomonas* using electroporation. The OFF–target effect were not investigated in *P. tricornutum*, it could have been great to have some results we can put in perspective with *Chlamydomonas*.

2.3. Nannochloropsis oceanica

Genus *Nannochlorospsis* is part of both *Heterokonta* superphylum and *Eustigamatophysceae* class. The *N. oceanica* strain used was IMET1. *Nannochloropsis* is used in the industry and can produce a broad range of products, from biofuels to high–value compounds [73]. Techniques like nitrogen deprivation can double the quantity of lipids inside *Nannochloropsis* [74, 75,]. The Xu research team decided to mutate the nitrate reductase [50], an enzyme converting nitrate to nitrite, based on the knowledge they previously acquired working on the *Nannochloropsis* genome and metabolism (As a consequence, the mutant will grow under ammonium supplementation but should not grow in medium containing nitrate only. The efficacy spread between 1 % and 0.1 %. As in *P. tricornutum*, they used a vector driven codon optimized Cas9–sgRNA production [76, 77] containing a Hygromycin gene using endogenous promoters and terminators. The sgRNA targets a PvuII enzymatic restriction site containing sequence within the nitrate reductase gene, as reported for Chlamydomonas [47], so they can use it in order to enrich the DNA pull with mutated DNA since the mutation is designed to destroy the enzymatic site.

The Cas9 is HA tagged but they failed to detect it by western blot analysis while both Cas9 mRNA and sgRNA were detected at good levels using qRT–PCR. Once again as shown for *C. reinhardtii* and *P. tricornutum* Cas9 protein level was not detected by western–blot analysis but Cas9

mRNA was easily measured. They also checked the functionality of the vector using fluorescence generated by the ble–mCherry protein, showing that the vector was indeed working.

The originality lays in the screening methods. 48 h post transformation, cells were plated in 10 hygromycin containing plates. 100 colonies were collected from each plate and pulled inside one flask. The subsequent culture (10 flask for 1000 colonies) lasted 14 days before DNA extraction and analysis. Wang, Q. et al. [50] and colleagues employed restriction–enzyme digested nested PCR called nPCR/RE fallowed by next generation sequencing. A part of extracted DNA underwent enzymatic pretreatment and the other not. Treatment of PCR product with PvuII restriction enzyme generate two bands (223 bps and 172 bps) corresponding to non–edited DNA and a 395–bp band corresponding to mutated DNA. Enzymatic pretreatment enrich your PCR amplicons in 395–bp bands. Among 10 flasks, the number 5 was the only one showing a clear 395–bp long band after gel electrophoresis. 97 % were wild type DNA without pretreatment whereas the perfect match frequency was up to 54 % using the pretreatment enrichment technique. Mutations occurred at the Cas9 cut site, a majority of them displaying a 5–bp deletion pattern at a frequency of 1.22 %, as expected those numbers increased up to 42.19 % using pretreatment.

We have to put those results in perspective because those efficiency percentages may concern flask number 5 only and not the whole group of 10 flasks. They estimated that the 5–bp deletion pattern account for 1 % since each flask were inoculated with 100 colonies. Said otherwise one colony may have been successfully transformed out of 100. Nevertheless, we have to be more careful here as they inoculated 10 flask with 100 colonies and flask number five only represent one tenth of the total amount of colonies picked up. Finally, the real efficiency percentage is perhaps 10 fold lower. The isolation and phenotyping were done from flask number five as well since it was the only one to provide satisfactory results. On the other side, the method (nPCR/RE) used to evaluate the technique rely on restriction site. As seen previously in *Chlamydomonas*, taking advantage of a restriction site to enrich a PCR pool in mutated DNA is nice, yet you may not be that lucky all the time. As a consequence, we have to think further about suitable techniques to check mutations and mutations efficiency independently of restriction site presence.

3. Discussion

In this paper, we review the strategies and statuses of the use of CRISPR technology to improve the efficiency of Eukaryotic microalgae to produce these biologically active compounds. Several studies aimed to explore Cas9 genome editing technique in microalgae. Nevertheless, we can conclude that there is some room for technical improvement if the scientific community aims to use the CRISPR/Cas9 system for further study. Cas9 protein toxicity issue needs to be addressed. Efficiency should be enhanced and comparable techniques and protocols are required in order to put results in perspective more easily. Mastering those parameters in some microalgae used as reference organism could lead to a more predictable and efficient use of the Cas9. To sum up; two main transfection techniques were used, electroporation and biolistic, and the Cas9–sgRNA complex can be present into the cell as a DNA–free ribonucleoprotein or expression vector.

Transformation technique used to deliver the Cas9 needs to be improved. Microalgae can be transformed using several techniques: protoplasts [79, 80, 81, 82, 83, 84, 85] glass beads [71], biolistic [71, 72, 73], electroporation [85] and agrobacterium [86, 87, 88], and scientists can target several organels like nucleus [89, 90, 91], mitochondria [92], and chloroplast [93, 90, 94, 95, 96, 97, 98]. Agrobacterium delivered CRISPR/Cas9 gave nice results in maize for example [36] but was not implemented for Cas9 delivery in eukaryotic microalgae. Trying other methods in order to transform microalgae is definitely something the scientific community has to do in order to choose the best one for each eukaryotic

Additionally, the insertion of DNA sequence from selection vector used in that transformation is a problem we need to unravel. In fact, all the publications only showed the capability to obtain a knock–out using the CRISPR technology but with pour efficiency; knock–out generation being only one CRISPR technique among others.

In order to control the mutation process, we need to insert a sequence of interest where we want, how we want. Point mutation like, Shin et al. [49] have shown were obtained unwittingly. Fortunately for them, the point mutation occurred at a strategical place, disrupting the activity of the enzyme. In order to have a better control of the knockout process we can insert a “stop–tag” inducing a frameshift, long enough to be seen by PCR, allowing a low cost genotyping and screening step before going forward with sequencing. “stop–tag insertion” could include or not specific sequence sequences in order to genetically “brand” the modified microalgae by the

industrial or the research institute: a genetic "foot–print" or "bar code" aimed to make the algae recognizable as part of a given project and property of a specific developer and/or owners. "Stop–tag" could also be used in conjunction with other "foot–prints" elsewhere in the genome in order to create another level of security by conjunction and data cross–checking. We propose and consider two alternative options over and above: a cell penetrating CRISPR/Cas9 and an inducible vector with tunable expression mode.

Cell penetrating CRISPR/Cas9 (CPP–RGEN or CPP–Cas9) was studied in human cells by Suresh Ramakrishna and colleagues [56] but it was also studied in TALEN and ZFN systems [100, 101]. The percentage of indel frequency was similar in the CPP–RGEN treated cell compared to plasmid delivery condition. We propose to use a cell penetrating peptide (CPP)–Cas9/sgRNA based strategy in order to transform some microalgae but also some cyanobacteria. Indeed translocation of FITC coupled CPPs were evaluated in *C. reinhardtii* [102] the results were encouraging, they also compared viability of cells using CPP with electroporated cells, showing that the later exhibited 80 % decrease in viability whereas CPP decrease in viability do not exceed 18 % only. Viability is also an important parameter since Cas9 toxicity is a recurring subject. Plasmid pVEC was the most efficient CPP tested in *Chlamydomonas* (TAT, PEN and TRA were also tested). The same system using cell-penetrating peptide can be applied in cyanobacteria as shown by Han–Jung Lee team [103, 104]. CPPs are not toxic for cyanobacteria, thus CPP–Cas9/sgRNA may provide in the future a fast and efficient way to modify both cyanobacteria and eukaryotic microalgae. CRISPR/Cas9 were also used in cyanobacteria. Expression vector driven Cas9 protein production was shown to be toxic as well in cyanobacteria in two independent studies [105, 106]. However, other papers showed that Cas9 could be used to do CRISPRi (CRISPR interference) using dead Cas9 [107, 108, 109].

Has we discussed herein, a precise tailoring is necessary if we want to reach the next level of genomic modification in eukaryotic microalgae. Then we can wonder if co–transfection of DNA, sgRNA and Cas9 protein using CPP can be done in microalgae, the purpose could be the insertion of a "stop–tag" DNA construct (ultramer like) with homologous wings allowing a precise insertion in the genome and a cost effective screening using PCR. Using the same method, we can tag protein and modify enzyme in a very clean manner. In order to support the feasibility of such project we draw your attention to the article published by [110], studying CPP–dsRNA in several eukaryotic microalgae such as *C. reinhardtii*, *C. vulgaris*, *P. tricornutum*, and *D. salina*.

Another path could be the design of a tunable vector with an antibiotic resistance cassette providing a first microalgae population harboring the vector in a "silent" mode. The Cas9 vector from the purified population can be "activated" by a drug, triggering the Cas9 mRNA and protein synthesis. The Rheoswitch system from the Intrexon Company can provide a dose dependent mechanism (https://www.dna.com/Technologies/RheoSwitch) so we would be able to set the precise dose to minimize toxicity and maximize indels frequency. To our knowledge such technique was used in human cell [111, 112, 113] and we propose here to use it in order to control Cas9 production and toxicity. Nevertheless, other system can be considered since several systems exist to trigger CRISPR editing and interference using, temperature, chemicals or light [114, 115].

4. Conclusion

Use of CRISPR technology need to be improved, robust protocol have to be found and time-tested. A particular attention should be paid to reproducibility: the scientific community have to use identical, or comparable, devices and protocols in order to draw valuable comparisons. Finally, it may be interesting to be more curious, or adventurous, testing the technique with other types of microalgae, so many are neglected.

5. Acknowledgements

The authors thank the support of CNRS UMR-EVS 5600, the National School of Mines of Saint Etienne and Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania".

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