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RESEARCH ARTICLE

Knockout of *Cia5* gene using CRISPR/Cas9 technique in *Chlamydomonas reinhardtii* and evaluating CO_2 sequestration in control and mutant isolates

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Abstract. CRISPR/Cas9 technology is one of the common methods of genome editing and targeted gene mutation, which has recently been used for manipulating microalgae such as *Chlamydomonas reinhardtii*. Besides, this technology can play a role in the fight against greenhouse gases (e.g., carbon dioxide) production by studying genetic pathways to improve algal strains. Among several genes in algae that respond to CO_2 and regulators control the expression of each; Cia5 is one of the most critical transcriptional regulators. In this research, we knocked out the *Cia5* gene using the CRISPR/Cas9 technique and analysed the ability of *C. reinhardtii* to perform CO_2 sequestration. Our results showed that *C. reinhardtii* has better performance (i.e., response to CO_2 treatment) in both control and mutant species at 0.5% CO_2 concentration than other concentrations. However, the difference between the control microalgae species and the mutant species was in the CO_2 removal efficiency. Additionally, our findings revealed that the control type isolate in CO_2 concentrations of 0.04%, 0.5% and 1% had removal efficiencies of 27%, 37% and 21%, respectively. Nevertheless, for mutant species in the same concentrations, the observed removal efficiencies were 16%, 23% and 9%.

Keywords. CRISPR/Cas9 technology; *Cia5* gene; CO_2 sequestration; *Chlamydomonas reinhardtii*.

Introduction

During the last two centuries, human beings have polluted the planet because of industrial revolution (Pianko-Oprych *et al*. 2016). As a result, we are facing the global warming, a critical crises in the last two decades (Davahli *et al*. 2020; Daryayehsalameh *et al*. 2021). CO_2 concentration in the atmosphere has increased from 280 ppm in the 18th century to 415 ppm in 2020 (Earth System Research Laboratory 2020). Algae have been considered as an efficient method of CO_2 sequestration (Molazadeh *et al*. 2019). Algae can produce more biomass by absorbing CO_2 (as a carbon source) and improve the photosynthesis process (Hankamer *et al*. 2007). One of the microalgae used in this field is *Chlamydomonas reinhardtii*. A considerable amount of literature has been published on the usage of these microalgae species on CO_2 sequestration (Farcas *et al*. 2012; Mortensen *et al*. 2015; Banerjee *et al*. 2021). Many aqueous photosynthetic organisms, such as *C. reinhardtii*, have a carbon concentrating mechanism (CCM) to provide the optimal concentration of carbon source for the Rubisco enzyme to improve photosynthetic activity. In algae, there are different genes for CO_2 fixation, and the expression of each of these genes can play an important role in CO_2 uptake (Fan *et al*. 2016). In various studies, the role of some of

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these genes, such as carbonic anhydrase, has received more attention (Basu *et al*. 2014; Swarnalatha *et al*. 2015). However, some regulatory genes, such as CIA5 (located in the cell nucleus), have received less attention. The *Cia5* gene exerts its effect on the deficit of carbon source (CO_2 and HCO_3^-) by improving the CCM mechanism activity. Moreover, *Cia5* is a transcription regulator that controls the expression of many genes, such as carbonic anhydrase that respond to low levels of CO_2 (Chen 2016). The expression of CCM-related genes is controlled by binding to a critical regulator called *Cia5* (CCM1) (Fang *et al*. 2012; Chen *et al*. 2017). This transcription factor has two zinc-finger motifs that make it bind to the target genes (Chen 2016). Therefore, evaluating the effect of this gene activity due to its targeted silencing is very effective (especially for further studies in the future). By knocking out this gene, its function as a master regulator in controlling the expression of various genes in response to CO_2 and CO_2 sequestration process can be evaluated.

Ci accumulation 5 regulator (*Cia5*) is a transcription factor for regulating the activity of CCM-related genes for CO_2 fixation, which is expressed at different concentrations of CO_2 (encoding transporters for Ci transport in plasma membranes). It is likely that posttranslational activities of this gene are required for its functions (Fang *et al*. 2012). This gene controls the expression of at least seven genes associated with CO_2 fixation (especially in low CO_2 conditions) and subsequently induces CCM activity (Fukuzawa *et al*. 2001). Thus, investigation of the effect of *Cia5* inactivation on CO_2 uptake is an interesting field of research.

Previous studies have reported the creation of target mutations in algae by various methods such as RNAi (Cerutti *et al*. 2011). In RNAi, an interfering RNA is used to silencing the target gene. However, it has been observed that the efficiency of this method is low in algae (i.e., there is the possibility of knocking out nontarget genes) (Banerjee *et al*. 2018). Recent evidence suggests that homologous recombination methods to knockout the target gene are more efficient compared to RNAi methods (Santiago *et al*. 2008; Hickey *et al*. 2011; Sharma *et al*. 2021). For this purpose, zinc finger nuclease (ZFN) (Sizova *et al*. 2013) and TALEN (Serif *et al*. 2017) methods were used for silencing the target gene. However, a major problem with this kind of methods is that there are still challenges regarding the adaptability of these two methods and targeted mutations. Another technique of knocking out target genes is clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9). Shin *et al*. (2016) showed that adaptability and targeted mutation are higher using this technique compared to previous techniques. The CRISPR/Cas9 system was identified to provide acquired immunity in many bacteria and archaea (Barrangou and Marraffini 2014; Rath *et al*. 2015). This RNA-based mechanism caused the cell to acquire resistance to foreign genetic elements such as bacteriophages and plasmids. It has been reported in the literature that it is possible to target the desired regions of the genomes of different organisms through the proper design of guide RNA by using the CRISPR technique (Gao and Zhao 2014; Manghwar *et al*. 2020; Owen *et al*. 2021).

What is not yet clear is the impact of *Cia5* regulator silence in *C. reinhardtii* on CO_2 sequestration process. This research aims to assess how the CO_2 sequestration process changes if the *Cia5* regulator is silenced in *C. reinhardtii*. The CRISPR/Cas9 construction is transferred directly to *C. reinhardtii* cells by electroporation. Guide RNA is also designed for the target region of the *Cia5* gene in algal species. Additionally, the expression of the target gene and the rate of CO_2 sequestration in control type and mutant strains are analysed and finally this study aims to optimize CRISPR construction transfer process in *C. reinhardtii* algae.

Material and methods

Microalgae culture

Chlamydomonas reinhardtii strain was cultured in a 3N-BBM liquid medium and after proper growth (stationary phase) samples were cultured on a 3N-BBM solid medium. Serial dilution was performed several times to purify with a ratio of 1:10, 1:100, 1:1000, and 1:10,000. Colonies formed on solid medium can be used as a purified sample. All samples were subsequently cultivated in 3N-BBM medium in 250 mL Erlenmeyer flasks medium. The growth conditions were kept constant for all the samples at a continuous aeration rate of 1 vvm and illumination (80 μmol photon m^{-2} s^{-1}) at 22°C. The illumination regime was 16h of light and 8h of dark for all the samples (Taher *et al*. 2014).

Target gene and sgRNA design

The *Cia5* gene coding sequence was prepared from the NCBI (accession number: XM_001699794.1). Then, the partial gene (exon 6 of the *Cia5* gene) was amplified with cDNA synthesized from the *C. reinhardtii* and 5'-TGGGTAGTGTGTATGTGCGT-3', 5'-CCCGGAATATGGTACCCCTC-3' primers. To extract RNA, add 1 mL of TRIzol buffer (Invitrogen LS12183555, USA) to the 50 mg wet biomass into a sterile 1.5 micro tube. The quantity and quality of the extracted RNA were evaluated with a spectrophotometer. Finally, the cDNA was synthesized using the reverse transcriptase enzyme according to the manufacturer's instructions (Qiagen). The amplified fragment was purified from gel electrophoresis and then sequenced to confirm the accuracy of the target gene (*Cia5*). To design sgRNA, CRISPR online construction design software was used. The gRNA sequence is as follows: CTGCTGAGTAGTAAGATGGG**GGG** (the highlighted part is the PAM sequence). This sequence was inserted into the expression vector pDe-CAS9 (see figure 1). In the c-terminal part of this sequence is the Cas9 protein. These sequences were

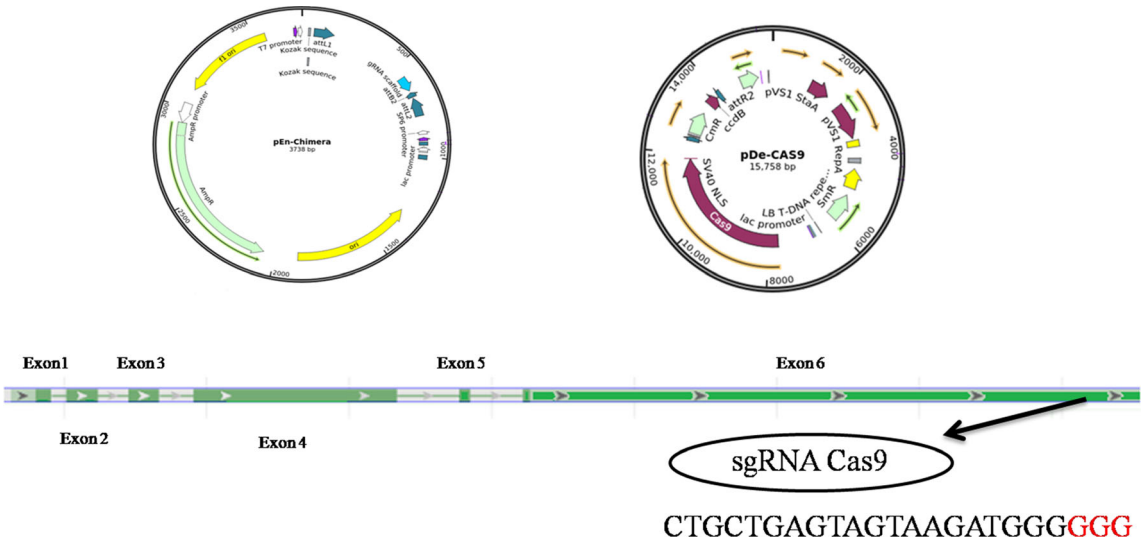

Figure 1. Gene constructs used in this study: CRISPR/Cas9 construction (pDE-Kan-Cas9) for knockout of *Cia5* gene. The *Cia5* gene has six exons that we designed sgRNA from exon 6.

synthesized by Toolgen Company (South Korea). After synthesis, they were dissolved in 50 μL of distilled water. The pEnChimera cloning vector was cleaved using the *Bbs*I restriction enzyme, and the guide RNA inserted into the vector using the T4 ligase enzyme. After confirming the correct placement of the fragment, gRNA was integrated by LR enzyme in the expression vector pDE-Kan-Cas9 (pDE-CAS9 derivative coding for sgRNA). Algal samples containing only Cas9 enzyme without targeted sgRNA were used as negative control. Chloramphenicol resistance (*cmr*) gene was also used as a positive control.

Transformation and selection of samples

In this study, fresh cells of *C. reinhardtii* were cultured at room temperature for 5 days under light conditions of 100-μmol m^{-2} s^{-1}. When the cell density reached 10^8 cells/mL, these cells were used to transfer the gene construction by electroporation (BioRad, USA) and 0.2 cm cuvettes. Five μL of the gene construct (CRISPR/Cas9 construction) was added to 35 μL of the algal suspension. Then, we added this suspension to the cuvettes of the electroporation device. The device settings were adjusted as follows: electrical resistance 550 Ω, porous pulse rate 250 V for 15 milliseconds and 10 polar pulses with an intensity of 20 V and pulse interval of 50 milliseconds.

After transferring the gene construct, cell suspension in the cuvettes was cultured in 10 mL of 3N-BBM medium with 50 mM sucrose. Samples were placed in low light conditions of 3-μmol $m^{-2}s^{-1}$ at 25°C. After 24 h, these samples were cultured on 3N-BBM solid medium with chloramphenicol (Cm) antibiotic (the pDE-Kan-Cas9 has resistance marker CMR). After one week, the colonies grown on solid medium were likely to contain the desired gene construct. Finally, DNA was extracted and samples were sent for sequencing.

Real-time PCR

Quantitative RT-PCR reaction was performed to evaluate the *Cia5* expression in wild and mutant *C. reinhardtii*. To do this reaction, TRIzol buffer (Invitrogen LS12183555, USA) was used, and the RNA extraction was performed. The cDNA was synthesized using the cDNA synthesis kit (Qiagen) and according to the manufacturer's instructions. The reaction mixture for each run was prepared with the SYBRGreen supermix (Bio-Rad). The reaction programme started with one cycle of 200 s at 95°C, followed by 40 cycles of the 20 s at 95°C, 20 s at 58°C and 20 s at 72°C. The primers used in this reaction are as follows:

5′-CGGAATATGGTACCCCTCTG-3′ and 5′-AGAAGGAATTAGGGTGAGGTCC-3′; 5′-GGTTAGGAGTGGCGAGCA-3′ and 5′-GCTGCGTTGGGAACTTCATT-3′

Evaluation of CO_2 sequestration

Control (wild) and mutant *C. reinhardtii* (containing pDE-Kan-Cas9) were grown in 5 L Erlenmeyer flasks containing 3N-BBM medium with effective volume of 2 L and light intensity of 80 μmol m^{-2} s^{-1}. Different concentrations of CO_2 (0.04%: air level, 0.5% and 1%) were bubbled with air into the medium. These concentrations were selected equal to the previous works in the literature for comparison purposes (Singh *et al*. 2011; Singh and Singh 2014; Mehta *et al*. 2019). Inlet and outlet concentration of the CO_2, CO_2 removal efficiency and dry weight, and the medium pH were measured daily. HEPES buffer was used to adjust pH to 8.5.

After 21 days (reaching the stationary phase), the results were completely recorded. The inlet and outlet CO_2 concentration was measured by a CO_2 sensor (Kimo AQ 100, France). The volume of inlet and outlet CO_2 were evaluated using a STAR GAS global diagnostics system. The CO_2 removal efficiency was calculated by the following equation:

$$CO_2 \text{ removal efficiency} = \frac{input - output}{input} \times 100 \quad (1)$$

The rate of CO_2 removal (RCO_2: $(\frac{gr \times L}{day})$ was calculated by the following equation (Jacob-Lopes *et al*. 2008):

$$R_{CO_2} = C \times P\left(\frac{M_{CO_2}}{M_c}\right). \quad (2)$$

Where C is the amount of carbon in algal biomass (weight/ %weight), P is biomass productivity $(\frac{gr \times L}{day})$ and M_{CO2} is the molecular mass of CO_2 and M_c is the atomic mass of carbon.

Equation 3 was also used for measuring CO_2 sequestration (gr) (De Morais and Costa 2007).

$$FC = (X_t - X_0)m_c V\left(\frac{M_{CO2}}{M_C}\right). \quad (3)$$

Here, FC represents the amount of CO_2 sequestration during the growth period (gr), X_t is the biomass concentration on day t $(\frac{gr}{L})$ and X_0 is the biomass concentration on day 0, m_c is the percentage of carbon in biomass $(\frac{gr}{L})$, and V is the volume of culture (L). These experiments were performed in three replications at three levels of CO_2 gas. The results were analysed by SPSS software. The mean values of P, F and adjusted R^2 for each experiment are reported.

Results

Cultivation of mutant C. reinhardtii on solid medium

Fresh cells of *C. reinhardtii* algae were cultured at 80 μmol m^{-2} s^{-1} light for 5 days at room temperature until the cell density reached 10^8 cells/mL. These fresh samples were used for electroporation. After the transfer, algal samples were cultured on a solid medium, and the desired colonies were collected (see figure 2a, b & c). DNA was then extracted from mutant *C. reinhardtii* strains and *Cia5* gene amplified using 5'-CGGAATATGGTACCCCTCTG-3' (binds internal to *Cia5* gene, upstream of *Cia5* CRISPR site) and 5'-AGAAGGAATTAGGGTGAGGTCC-3' (binds internal to *Cia5* gene, downstream of *Cia5* CRISPR site) primers. The expected size product (~290 bp) was observed in the mutant *C. reinhardtii* (see figure 2d). These fragments (in wild and mutant *C. reinhardtii*) were purified with the DNA gel extraction kit (Bioneer, Korea), and samples were sent for sequencing. The results showed that the knockout occurred in the target area (see figure 2e).

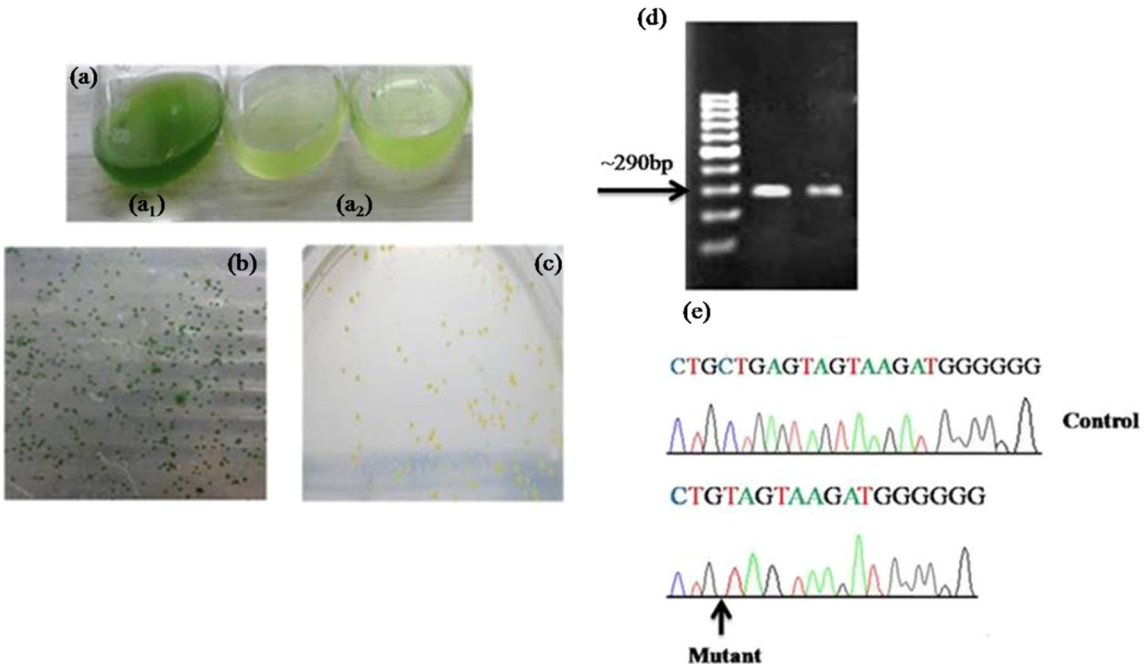

Figure 2. Growth of control and mutant *C. reinhardtii* in (a) liquid,and (b–c) solid media. (a_1) and (a_2): *C. reinhardtii* grown before the transfer of the CRISPR vector with bold green colour; algae grown before (b) and after (c) the transfer of the CRISPR vector with yellowish green colour. (d) Digestion of the target fragment for evaluation of Cas9 activity, expected amplicon ~290 bp. (e) Sequencing of samples and mutation at the target site.

Real-time PCR

The results of this study showed that ~4 to 7-fold changes in mRNA abundance of *Cia5* in wild strain. On the other hand, the mRNA abundance of mutant strain was 0.5 to 1-fold in different CO_2 concentrations (see figure 3). Expression ratio showed significant changes in mutant strain. As previously mentioned, *Cia5* was always responsive to the studied CO_2 concentrations; *Cia5* tries to promote the whole process to enhance CO_2 sequestration by the cells. Increasing the expression of this gene at wild strains lead to improved activity of the Ci transport pathway to continue cell growth under resource shortages.

Determination of CO_2 sequestration in control and mutant C. reinhardtii

Control and mutants *C. reinhardtii* were cultured in 3N-BBM medium under 0.04%, 0.5% and 1% CO_2 concentrations in three repetitions. CO_2 sequestration was assessed after 21 days according to the method described in evaluation of CO_2 sequestration section in Materials and Methods. The results showed that control isolates at concentrations of 0.04%, 0.5% and 1% of CO_2 had removal efficiencies of 27%, 37% and 21%, respectively. Also, in these concentrations, the sequestration rate was equal to 1.15, 1.19 and 1.14 gL^{-1} per day, respectively. However, for mutant isolates at the same concentrations, the removal efficiencies were 16%, 23% and 9%, respectively. In addition, the CO_2 sequestration rate was equal to 0.85, 1.03 and 0.73 gL^{-1} per day, respectively. Finally, the amount of CO_2 sequestration during the growth period (after 21 days) at the three concentrations mentioned above was examined. The results showed that this amount was equal to 53.65, 84.75 and 47.23 g for control isolates and 37.19, 61.09 and 20.89 g for mutant isolates. The results are summarized in table 1. The model F-value is equal to 90, 84 and 155.02 for CO_2 removal efficiency, CO_2 removal rate, and CO_2 sequestration, respectively, indicating that the used model is significant (sig = 0.000 for all experiments). The P value was < 0.01 in these experiments; adjusted $R^2 = 0.974$, 0.972 and 0.984 for CO_2 removal efficiency, CO_2 removal rate and CO_2 sequestration, respectively.

In general, the results showed that the CO_2 sequestration process occurs better at 0.5% CO_2 concentration. Moreover, the results indicate that mutant isolates where the *Cia5* gene was knocked out, the CO_2 sequestration process was significantly reduced compared to the control isolates.

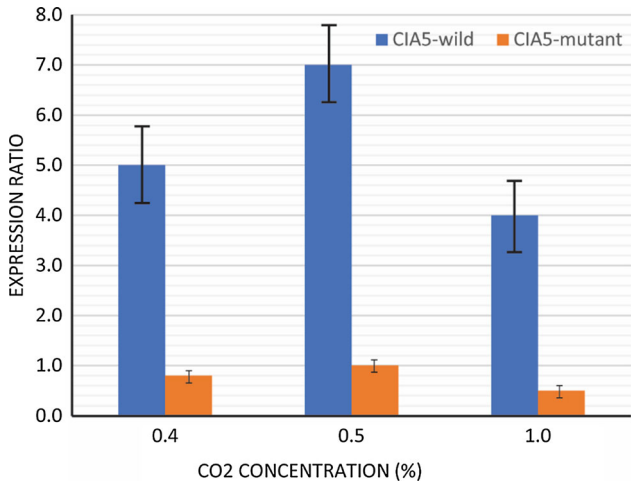

Figure 3. Quantitative real-time PCR analyses of *Cia5* gene expression in wild and mutant strains of microalga *C. reinhardtii* for CO_2 sequestration.

Discussion

In this study, we successfully transferred the CRISPR/Cas9 construct to the *C. reinhardtii* isolate. To date, there have been several reports of the transfer of CRISPR construct to the algae, such as *C. reinhardtii* as a model organism (Baek *et al*. 2016; Greiner *et al*. 2017; Guzman-Zapata *et al*. 2019). There are several studies on CRISPR gene editing in *Chlamydomonas* sp. However, evaluating the activity of genes associated with CO_2 fixation, especially with the CRISPR technique and editing of genes involved in the CO_2 sequestration process with the CRISPR technique have not been vastly studied in the literature. Therefore, to the best of our knowledge, the present study is one of the first reports of transient expression of a CRISPR construct in *C. reinhardtii* and evaluating the function of a factor regulating genes expression (Cia5) CO_2 sequestration. mRNA and the protein of this regulator are constantly present in algal cells, and even this presence can lead to posttranslational modification of *Cia5* that controls the synthesis and expression of CCM components (Xiang *et al*. 2001; Farcas *et al*. 2012). This process is vital in how the algae respond to low CO_2 conditions (Xiang *et al*. 2001). Also, this gene is involved in the mechanism of the CCM and

Table 1. The ability of control and mutant strains of microalga *C. reinhardtii* for CO_2 sequestration.

Treatment (CO_2 concentration% + algal strain)	CO_2 removal efficiency (%)	CO_2 removal rate (gL per day)	CO_2 sequestration (gr)
0.04% + control	$27\% \pm 1.3^{b*}$	1.15 ± 0.1^{a}	53.65 ± 2.7^{c}
0.04% + mutant	$16\% \pm 0.9^{d}$	0.85 ± 0.04^{c}	37.19 ± 1.9^{e}
0.5% + control	$37\% \pm 1.1^{a}$	1.19 ± 0.09^{a}	84.75 ± 3.1^{a}
0.5% + mutant	$23\% \pm 1.1^{c}$	1.03 ± 0.07^{b}	61.09 ± 2.4^{b}
1% + control	$21\% \pm 1.4^{c}$	1.14 ± 0.05^{a}	47.23 ± 2.1^{d}
1% + mutant	$9\% \pm 1.2^{e}$	0.73 ± 0.03^{d}	20.89 ± 1.8^{f}

*The significance level is equal to 0.01. Same superscript are not significantly different from each other.

the improvement of cell photosynthesis at low external CO_2. By designing Cia5-sgRNA, we inserted a mutation in the target gene; the results showed that *Cia5* could play an essential role in improving microalgal activity and CO_2 sequestration. After transferring the gene construct to microalgae and culturing it in solid medium, the yellow/green colour of mutant colonies was observed compared to the control type (figure 2c). This is due to changes in the photosynthetic apparatus involving a decreased chlorophyll content and lack of access to sufficient carbon supply. *Cia5* gene function and its effect on photosynthetic apparatus have been reported in various studies (Im and Grossman 2002; Pollock *et al*. 2003; Wang *et al*. 2005).

Also, in many studies, it has been observed that direct transfer of a *CRISPR* gene construct without a donor vector leads to nontarget mutations in the cell (Ma *et al*. 2015; Moreb *et al*. 2020). Therefore, in this study, we used homologous recombination methods and a donor vector for targeted mutation. This process led to greater accuracy in target gene mutation. For further studies, it is suggested that this construct be transferred to other microalgae to evaluate the function of other genes involved in the CO_2 sequestration pathway. We can study the effect of different genes and use them to reduce pollutants (such as CO_2) in different environments.

Another important point is that the exact locus of the gene construct and its effect on specific nucleotides is unknown. Therefore, with each transfer of the construct and sequencing, different mutations appear. The mutation site in the positive samples is inside the target gene but at different loci. This could be due to errors that occur due to the transfer of the construct by the electroporation method. This has been expressed in other studies (Hiruta *et al*. 2018). Therefore, phenotypic assessments play a significant role in evaluating mutant isolates.

In conclusion, the use of fossil fuel leads to an increase in environmental pollutants such as CO_2 in the atmosphere and hydrosphere. Biological sequestration of pollutants can have positive effects in reducing the destructive effects of pollutants. Microalgae such as *C. reinhardtii* can be helpful in this regard. To date, many studies have been conducted in this field, but the use of novel methods to evaluate the molecular properties of microalgae to respond to greenhouse gases is significant. Therefore, using CRISPR/Cas9 technique as a suitable method for targeted mutation can help study different genes' functions. On the other hand, since microalgae have a set of genes to respond to environmental pollutants, by evaluating the function of each gene, the importance of the target gene can be investigated, and valuable genes can be isolated from algae by cloning methods. Also, there are several papers on *CRISPR* gene editing in *Chlamydomonas* sp, but it is noteworthy that so far, there have been a very few studies evaluating the activity of genes associated with CO_2 fixation, especially with the CRISPR technique. Overall, our literature review highlighted the need for study on the editing of genes involved in the CO_2 sequestration process with the CRISPR technique.

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