THINK INDIA JOURNAL

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

Genetically Engineered Diatoms For The Enhanced Production of Green Fuels

S.M.Savini Gunarathne[1] and Shristy Gautam[1*]

[1]Department of Molecular biology and Genetic Engineering, School of Bio Engineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India, 144411.

*Corresponding author email id: shristy.23772@lpu.co.in

Abstract

Biofuels derived from renewable, eco-friendly biological sources are the ultimate hope of researchers as a remedy for the depletion of biofuels. Depletion of fossil fuels is one of the most heeding issues emerging at present. But not only that, but also other bigger issues such as increased emission of greenhouse gasses leading to global climatic change are also threatening to the existence of living beings. So biofuels play a major role here contributing to the mitigation of global climatic change by reducing the release of greenhouse gasses and also it provides the best solution for the depletion of fossil fuels. Moreover, when considering all the sources that can be utilized for the production of green fuels, diatoms as a part of the micro algae family serve an important place. These have the ability to grow in any kind of water resource including sewage water in large amount that is enough to provide a supply meeting the emerging demand. In addition to that accumulation of lipids inside the cells and the ability to convert this oil into refined diesel and many other fuels such as gasoline make the diatoms more significant. However, in order to use the diatoms to obtain the desired uses in the required amounts mainly as a source of biofuels, it requires modifications of the improvements of the diatom genome. Modern genome editing tools such as RNAi, FOK I-I, TALENS, meganucleases, CRISPR associated Cas 9 help in accomplishing these modifications making the site specific editing of the diatoms possible. These genetic engineering techniques deliver the advantages of more accuracy, efficiency, simplicity in the improvement of diatom genome than any other conventional methods. This review paper provides an insight into the types of modifications that can be done to improve the biofuel production from diatoms and will provide insight into their adaptive capacity and are a prerequisite for metabolic engineering.

Keywords: Diatom, Biofuel, RNAi, Triacylglyceride

1. Introduction

Due to the increasing human population, the limited availability of fossil fuels and most importantly to reduce the environmental pollution caused by the burning of fossil fuels, the development of green fuels or in other words the eco friendly, cost friendly, renewable biofuels derived from biological sources has become one of the priorities of current researches. The global climatic change is one of the major issues that are caused by the burning of fossil fuels as traffic is a major form of releasing greenhouse gases into the atmosphere. In the United Kingdom, transportation contributes to more than a fifth of the total greenhouse gas emissions. So biofuels provide a solution for this, reducing the emission of greenhouse gases. Diatoms as a promising biological source of fuel related molecules such as lipids comes into the picture

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

overpowering many other alternative sources of biofuels due to many causes. Diatoms or the micro algae delivers the advantage of eliminating the competition for land that are being used for the food as they can be grown from sewage water to any kind of water without any effort from our side. The ubiquitous presence of diatoms as well as the characteristic of growing rapidly and double their biomass in a few hours is another advantage. The growth of diatoms can be easily manipulated by the availability of silicate and also almost all of their biomass can be used profitably [1]. All these reasons make the diatoms more significant among all other renewable resources available for the green fuel production and allow the researchers to focus into more improvements of their genome so as to harvest the maximum possible from the biomass. Nuclease guided genome editing tools such as RNAi, FOK I-I, TALENS, meganucleases, CRISPR associated Cas 9 make the improvement of genome of different organisms such as diatoms to enhance the production of this lipid content possible, facilitating the production of biofuels. Diatom species such as *Thalassiosira pseudonana, Phaeodactylum tricornutum, Cylindrotheca fusiformis, Pseudonitzschia* sp., *Fragilariopsis cylindrus* are used as model organisms in most of these attempts [2]. The genetic manipulations in the diatom genome are done in several ways. In order to enhance the lipid content in the cells approaches such as inhibiting the function of genes coding for lipids, lipid biosynthesis pathways modification, can be followed. These techniques are more precise, fast, reliable and simpler than the traditional methods such as strain discovery and selection process for finding lipid rich species. This review paper provides an overview about the genetic modification methods that has been followed targeting the enhanced production of biofuels.

2. **Different approaches for the genetic engineering of the diatoms**

2.1 Decreasing the lipid catabolism using TALEN-based targeted mutagenesis

Acyl-CoA molecules in diatoms are the sources for biofuel production. These molecules are used by many lipid metabolic enzymes such as endogenous thioesterases and the enzymes hydrolyze those molecules to form fatty acids. The availability of acyl-CoA is a major factor of membrane lipids and storage lipids quantity in diatoms [3]. In eukaryotic microalgae, TAG biosynthesis occurs in two main metabolic pathways as an acyl-CoA-dependent pathway and acyl-CoA-independent pathway. In acyl-CoA-independent pathway, the fatty acyl moiety is converted to TAG by the enzyme phospholipid diacylglycerol acyltransferase (PDAT). This is one of the major enzymes involved in the enhanced lipid production. One approach for obtaining a higher content of triacylglycerols in the diatom cells is to engineer the diatom cells to inhibit the hydrolysis of triacyglycerols. This thioesterase enzyme is involved in the hydrolysis of long and medium-chain fatty acyl-CoAs, but it shows most affinity towards the mono-unsaturated and long-chain saturated fatty acyl-CoAs. The following method has been followed in Hao et al. [3] for the inactivation of the ptTES1 gene in *Phaeodactylum tricornutum.* By fusing amino acids to eGFP, ptTES1 gene were localized and it was observed that ptTES1-eGFP construct was expressed by *P. tricornutum* and were identified using GFP fluorescence. Then the ptTES1 knockout mutants were constructed using TALEN [4]. TALEN and knockout plasmids were transformed into *P. tricornutum* cells simultaneously. PCR screening was applied to screen the

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

positive ptTES1 knockouts. Complete prohibition of the ptTES1 expression in those positive knockouts [5] and knockouts were selected with the help of zeomicin. Algal cultures (2×10^5 cells/ ml) were inoculated and sampling was collected every two days and cell number, nitrate concentration using spectrophotometer at 220 nm [6] and neutral lipid content were measured. Nile red dye was assayed using fluorometric assay to define and monitor the neutral lipid content and was measured at 572 nm [6].

According to the results obtained in [3] during day 4 to 10, the knockout line showed slower growth than wild type, almost the same rate of nitrogen use. Amount of neutral lipid diatom cells, which was corresponding to fluorescence, measured using the Nile red staining was highest in the knockout line. The fluorescent staining Bodipy505/515 of diatom cells grown on f/2 media deprived with nitrogen was used to sustain the relationship between the inactivation of ptTES1 and the lipid production. The diameter of the lipid droplets of the transformed cells showed a significant increase in the transforming cells with the nitrogen deprivation. Deactivation of the native ptTES1 gene was grown on nitrogen-replete media and resulted in increase in TAG content.

2.2. Use of antisense and RNAi approaches for the targeted knockdown of a multifunctional lipase

The desired knockout of Thaps3_264297 in *T. pseudonana* has been carried out in the following manner in Trentacoste et al. [7]. The constructs, pA78cgi encoding an antisense and pI1001cgi encoding RNAi were designed targeting a portion of the gene Thaps3_264297 and transformed using microparticle bombardment method into *T. pseudonana*. PCR was used to confirm the presence of integrated constructs. For the expression of pA78cg, fcp protein promoter was responsible, while an inducible nitrate reductase promoter controlled the expression of pI1001cgi. Then the designed constructs were changed into wild type *T. pseudonana*. Then the transformants were checked for lipid accumulation and growth with both well nutrients supplied and deplete conditions [7]. For lipid analysis method of Folch et al [8] was followed to extract the lipids [9]. The wild type *T. pseudonana* found to accumulate more TAG under silicon limited conditions. To determine the effect of the inactivation of the gene on the lipid accumulation imaging with flow cytometry. So metabolic manipulations achieved through genetic engineering techniques can be applied to increase lipid content in diatoms without affecting their growth.

2.3. **Modification of the lipid biosynthesis pathway**

Glycero-phospholipids are important functional and structural components of cellular membranes in cells. Furthermore, they take on a central role as energy and deliver messages in signal transduction. Glycerol 3-phosphate is the first molecule in De novo pathway. The final step in triacylglyceride (TAG) biosynthesis is catalyzed by Diacylglycerol acyl-transferase it is called the rate-limiting enzyme. Moreover, it is highly studied enzymes in the entire acyl lipid metabolism due to its importance. Also micro algae, including diatoms are unique due to the presence of several DGATs [10]. The overexpression of DGAT2 enzyme results not only in the increased neutral lipid content, but also its increased mRNA expression. As explained in Niu et al. [11], the DGAT2 gene was cloned between the promoter fcpC and terminator fcpA of the fcp

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

gene of *P. tricornutum* and then the construct pHY31 was transformed. To enhance its translation, the omega leader sequence and "ACC" nucleotide motif were added before DGAT2. The transformant diatom cells were selected using chloramphenicol selection. PCR was carried out to screen the transformed *P. tricornutum*. In terms of the biomass accumulation, the transgenic diatoms showed a growth similar to wild type in f/2 medium without antibiotic chloramphenicol. Transgenic diatom cells showed an increase in neutral lipid content up to 35% increase when measured by Nile red fluorescence staining.

3. Discussion

In microalgae such as diatoms over-expression of enzymes malic enzyme [12], glycerol-3-phosphate acyltransferase [13], and diacylglycerol acyltransferase [14], have been shown to be effective in enhancing lipid accumulation. In addition to those modifications of the biosynthetic pathways of lipid synthesis, inhibition of the expression of the genes coding the enzymes involved in the catabolism was also found to be effective in the enhancing the lipid content in diatom cells. Apart from the experimentation that is reported in this paper regarding the inhibiting the function of genes coding for the lipid catabolizing enzymes, inactivation of the UDP-glucose pyrophosphorylase gene has been also resulted in the enhanced accumulation of lipids in *Phaeodactylum tricornutum* [15]. Also, mutations have been induced in the ADP-glucose pyrophosphorylase gene of *Chlamydomonas reinhardtii* [16]. Similarly, in all these attempts the lipid content in the cells was increased using different strategies. Moreover, some metabolic engineering efforts have been concentrated on the positive consequence of bottling up the degradation of substrate to bring up the levels of synthetic and natural products.

4. Conclusion

These genetic engineering techniques that have been briefly reported in the paper results in the enhanced lipid content in cells supported by the mentioned previous studies. TALEN-based genome editing technique that was employed for the disturbance of this acyl-CoA thioesterase gene resulted increase in TAG. But it did not show any change in the other phosphoglycerolipids and galactoglycerolipids. Most importantly, this property of producing increased content of lipids of the engineered or mutated diatom strain will be there for more than 1 yr.. Moreover the RNAi approach also resulted in the successful knockdown of the multifunctional enzyme, thereby increasing the lipid content. Therefore, all these results confirm that genetic engineering approaches are capable of inducing successful mutations resulting increased lipid accumulation in diatoms that will ultimately contribute to the production of biofuels.

5. References

1. J.K. Wang and M. Seibert, "Prospects for commercial production of diatoms," Biotechnology for Biofuels, 10, pp. 16, 2017.

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

2. W. Huang and F. Daboussi, "Genetic and metabolic engineering in diatoms," Philosophical Transactions of the Royal Society B: Biological Sciences, 372, pp. 20160411, 2017.
3. X. Hao, L. Luo, J. Jouhet, F. Rébeillé, E. Maréchа, H. Hu, Y. Pan, X. Tan, Z. Chen, and L. You, "Enhanced triacylglycerol production in the diatom *Phaeodactylum tricornutum* by inactivation of a Hotdog-fold thioesterase gene using TALEN-based targeted mutagenesis," Biotechnology for biofuels, 11, pp. 312, 2018.
4. P.D. Weyman, K. Beeri, S.C. Lefebvre, J. Rivera, J.K. McCarthy, A.L. Heuberger, G. Peers, A.E. Allen, and C.L. Dupont, "Inactivation of *Phaeodactylum tricornutum* urease gene using transcription activator-like effector nuclease-based targeted mutagenesis," Plant biotechnology journal, 13, pp. 460-470, 2015.
5. M.A. Quilliam, G.M. Hallegraeff, D.M. Anderson, and A.D. Cembella, "Chemical method for lipophilic shellfish toxins," Manual on harmful marine microalgae, pp.211-245, 2003.
6. F. Ge, W. Huang, Z. Chen, C. Zhang, Q. Xiong, C. Bowler, J. Yang, J. Xu, and H. Hu, "Methylcrotonyl-CoA carboxylase regulates triacylglycerol accumulation in the model diatom *Phaeodactylum tricornutum*," The Plant cell, 26, pp. 1681-1697, 2014.
7. E.M. Trentacoste, R.P. Shrestha, S.R. Smith, C. Glé, A.C. Hartmann, M. Hildebrand, and W.H. Gerwick, "Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth," Proceedings of the National Academy of Sciences, 110, pp. 19748-19753, 2013.
8 J. Folch, M. Lees, and G.H.S. Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," Journal of biological chemistry, 226, pp. 497-509, 1957.
9. W.C. William, "Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids," Pergamon Press, 1982.
10. J. Oakes, D. Brackenridge, R. Colletti, M. Daley, D.J. Hawkins, H. Xiong, J. Mai, S.E. Screen, D. Val, and K. Lardizabal, "Expression of fungal diacylglycerol acyltransferase2 genes to increase kernel oil in maize," Plant physiology, 155, pp. 1146-1157, 2011.
11. Y.F. Niu, M.H. Zhang, D.W. Li, W.D. Yang, J.S. Liu, W.B. Bai, and H.Y. Li, "Improvement of neutral lipid and polyunsaturated fatty acid biosynthesis by overexpressing a type 2 diacylglycerol acyltransferase in marine diatom *Phaeodactylum tricornutum,"* Marine drugs, 11, pp. 4558-4569, 2013.
12. J. Xue, Y.F. Niu, T. Huang, W.D. Yang, J.S. Liu, and H.Y. Li, "Genetic improvement of the microalga *Phaeodactylum tricornutum* for boosting neutral lipid accumulation," Metabolic engineering, 27, pp. 1-9, 2015.
13. Y.F. Niu, X. Wang, D.X. Hu, S. Balamurugan, D.W. Li, W.D. Yang, J.S. Liu, and H.Y. Li, "Molecular characterization of a glycerol-3-phosphate acyltransferase reveals key features essential for triacylglycerol production in *Phaeodactylum tricornutum*," Biotechnology for Biofuels, 9, pp. 60, 2016.
14. N.N. Zulu, J. Popko, K. Zienkiewicz, P. Tarazona, C. Herrfurth, and I. Feussner, "Heterologous co-expression of a yeast diacylglycerol acyltransferase (ScDGA1) and a plant oleosin (AtOLEO3) as an efficient tool for enhancing triacylglycerol accumulation

ISSN: 0971-1260
Vol-22-Issue-16-August-2019

in the marine diatom *Phaeodactylum tricornutum*," Biotechnology for Biofuels, 10, pp. 187, 2017.

15. F. Daboussi, S. Leduc, A. Maréchal, G. Dubois, V.r. Guyot, C. Perez-Michaut, A. Amato, A. Falciatore, A. Juillerat, and M. Beurdeley, "Genome engineering empowers the diatom *Phaeodactylum tricornutum* for biotechnology," Nature communications, 5, pp. 3831, 2014.
16. V.H. Work, R. Radakovits, R.E. Jinkerson, J.E. Meuser, L.G. Elliott, D.J. Vinyard, L.M.L. Laurens, G.C. Dismukes, and M.C. Posewitz, "Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains," Eukaryotic cell, 9, pp. 1251-1261, 2010.