Genetic Engineering Tools for Enhancing Lipid Production in Microalgae

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1 Introduction

Microalgae have been of considerable interest in the last decade as a possible resource for biofuel production, owing to its potential for high biomass generation rates compared to terrestrial plants (Radakovits et al. 2010). Many microalgal species are reported to produce substantial amount of storage lipids (triacylglycerols) under stress conditions, which could be effectively utilized for the production of biofuels. However, naturally growing microalgae might not be able to provide enough resources for the production of renewable biofuels so as to replace fossil transportation fuels (Li et al. 2008). To date, numerous research work have been performed to improve the lipid accumulation in microalgae which includes modifying the cultivation conditions such as nutrient stress, light and temperature.

Currently, several efforts are being made globally to increase the storage lipid accumulation in microalgae, by employing methods other than improving cultivation conditions. One potential approach to enhance lipid production is by employing genetic engineering (GE), based on the key enzymes involved in lipid biosynthesis. Many studies have been carried out in the recent past to understand the basic lipid metabolism of different microalgal strains. With the advent of advanced molecular techniques, the complete nuclear and chloroplast genome sequencing of many microalgal species is currently available. A basic knowledge of the key genes present in microbial genome would assist researchers in the manipulation of algal genomes more specifically and would form a strong platform for the future GE strategies in microalgae.

Microalgal GE for enhanced lipid production has mainly focused on manipulating the key genes associated with lipid synthesis through forward or reverse genetics (Radakovits et al. 2010; Hlavová et al. 2015). In this chapter, we focus briefly on the lipid and fatty acid biosynthesis pathways in microalgae and the common genetic engineering tools that have been employed to enhance lipid production. Some of the challenges affecting microalgal genetic engineering research have also been discussed in this chapter.

2 An Overview of Lipid Biosynthesis Pathways in Microalgae

Arabidopsis is considered as a model organism for understanding the enzymes involved in lipid synthesis of all photosynthetic organisms. Based on the sequence homology of genes involved in lipid metabolism of microalgae and Arabidopsis, it was generally assumed that microalgae have similar lipid metabolic pathway as that of higher plants. However, after the whole genome sequencing of *Chlamydomonas*, it was established that microalgae have simple lipid metabolic pathway compared to higher plants. Biosynthesis of fatty acids in microalgae occurs in the chloroplast and is regulated by an enzyme complex acyl carrier protein (ACP) fatty acid synthase (FAS) type 2 (Harwood and Guschina 2009). FAS enzyme is a polypeptide chain with multiple domains, each having distinct enzyme activity required for fatty acid biosynthesis. The first step in fatty acid biosynthesis is the formation of malonyl-CoA from acetyl CoA, catalysed by acetyl-CoA carboxylase (ACCase) (Blatti et al. 2013). In the chloroplast, photosynthesis provides an endogenous source of acetyl CoA, and more than one pathway may contribute in maintaining the acetyl CoA pool. ACCase is considered as the main enzyme that catalyses

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B. Singh et al. (eds.), *Algae and Environmental Sustainability*, Developments in Applied Phycology 7,
DOI 10.1007/978-81-322-2641-3_10

the first step reaction in lipid biosynthesis. It has three major functional sites that promote the formation of malonyl-CoA, the main carbon donor for fatty acid biosynthesis. This is considered as a rate-limiting step in lipid synthesis which involves two stages: (a) the transfer of CO_2 (HCO_3^-) by biotin carboxylase portion of ACCase to a biotin prosthetic group that is attached to the E-amino group of lysine residue and (b) the transfer of activated CO_2 from biotin to acetyl CoA. Production of acetyl CoA and its conversion to fatty acid, however, depends on factors such as tissue type, light/dark condition, developmental stages and species (Thelen and Ohlrogge 2002).

Biosynthesis of fatty acid is a multistep reaction which involves condensation, reduction, dehydration and reduction. A malonyl group is first transferred from CoA to a protein cofactor acyl carrier protein (ACP) which is involved in the whole process until the formation of a 16- to18-carbon product, ready to be transferred either to the glycerolipids or exported from the plastid. The next step is a condensation reaction. At this stage, the malonyl group of malonyl-ACP undergoes a series of condensation reactions with acetyl CoA releasing CO_2, that helps to drive the reaction forward. At least three groups of condensing enzymes (commonly called 3-ketoacyl-ACP synthase) are involved in the synthesis of 18-carbon fatty acids (Hu et al. 2008). The initial condensation reaction leads to the formation of a four-carbon product, 3-ketoacyl-ACP, catalysed by 3-ketoacyl-ACP synthase III (KASIII). Other condensation enzymes are KASI which is thought to be involved in the production of 6- to 16-carbon chain products and KASII for the elongation of 16-carbon ACP to stearoyl-ACP. This reaction cycle is completed by a reduction reaction catalysed by enoyl-ACP reductase. Each cycle of these four reaction steps lengthens the fatty acid precursor chain by 2 carbons while still attached to ACP as a thioester (a process known as elongation) leading to the formation of saturated 16:0-ACP and 18:0-ACP. Unsaturated fatty acid is formed when a double bond is introduced by the enzyme stearoyl-ACP desaturase. This is followed by a reduction process where 3-ketoacyl-ACP is reduced at the carbonyl group by the enzyme 3-ketoacyl-ACP reductase and then dehydration by the hydroxyacyl-ACP dehydratase. Elongation of a fatty acid chain takes place in the plastid and continues until the acyl group is removed from ACP, either as a result of hydrolysis of acyl-ACP by acyl-ACP thioesterase, releasing free fatty acid, or by the transfer of fatty acid from ACP to glycerol-3-phosphate or to monoacylglycerol-3-phosphate by one of the two acyltransferases (Hu et al. 2008) (Fig. 10.1).

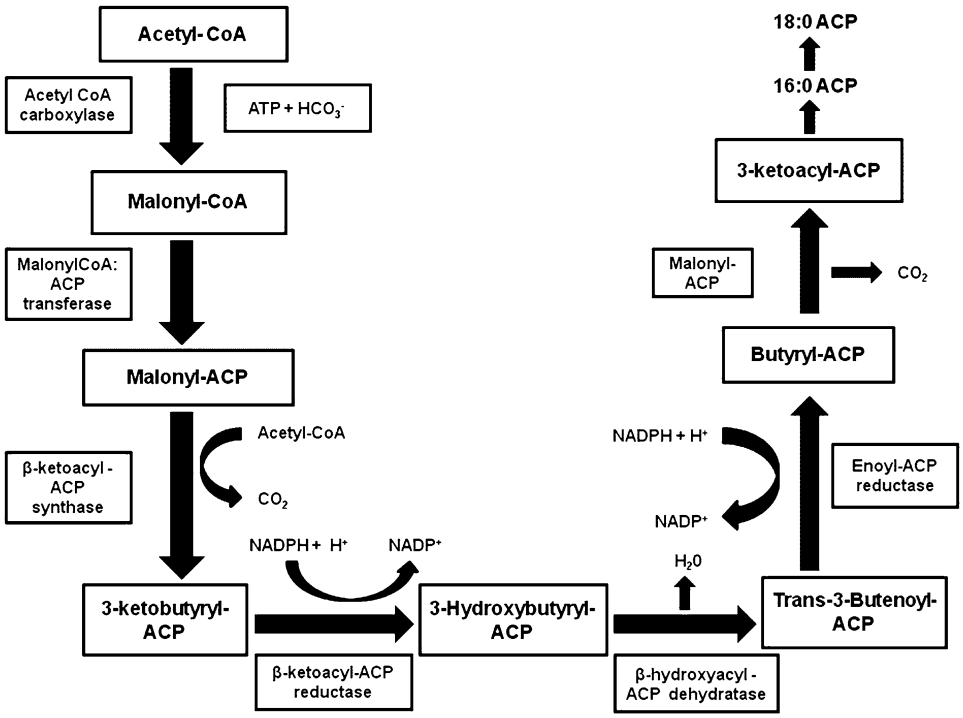

Fig. 10.1 Fatty acid biosynthesis pathway in microalgae

3 TAG Biosynthesis in Microalgae

The triacylglycerols (TAGs) are the storage form of metabolic energy in microalgae and various other organisms. Biosynthesis of TAGs starts with the two precursors, l-α-phosphoglycerol and acetyl CoA (Yu et al. 2011). Initially, the fatty acids synthesized in chloroplast are exported to cytosol where acylation reactions take place and the synthesized fatty acid is transferred to glycerol-3-phosphate to form phosphatidic acid (PA). This reaction is catalysed by the enzyme glycerol-3-phoshpate acyltransferase. In the next step, diacylglycerol (DAG) is produced by dephosphorylation of PA in the presence of phosphatidic acid phosphatase (Fig. 10.2). Synthesis of TAG is completed by the attachment of fatty acid to DAG in the presence of the enzyme diacylglycerol acyltransferase (Merchant et al. 2012). The acyltransferases involved in TAG synthesis may exhibit preference for specific acyl-CoA molecules which could be an important factor in determining the acyl composition of TAG. For example, the lyso-PC acyltransferase in *Nannochloropsis* cells prefers 18:1-CoA over 16:0-CoA (Roessler et al. 1994). Although lipid biosynthesis in algae has been assumed to share a similar pathway as that of higher plants, the annotation of chloroplast genes in microalgae has shown differences in the gene sequences. For example, gene annotation of *C. reinhardtii* has shown some differences in the type of gene families related to lipid biosynthetic pathways (Riekhof et al. 2005). Therefore, knowledge of the genes involved in the synthesis of fatty acids and TAGs specific to microalgae would allow for the improvement of GE technology for enhanced lipid production.

4 Genetic Engineering Approaches to Improve Lipid Synthesis in Microalgae

Various genetic engineering systems have been developed for strain improvement in different algal species. They are broadly classified into two main categories, viz. reverse and forward genetics. Reverse genetics involves the application of traditional random (chemical/physical) mutagenesis, whereas forward genetics involves identification of a particular gene of interest and manipulation of its expression level.

4.1 Forward Genetics Approach: Random and Insertional Mutagenesis

Chemical and physical mutagens are among the most widely used traditional methods for strain improvement. The most commonly used chemical mutagens are alkylating agents such as ethyl methanesulfonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (Table 10.1). They were the first chemicals used for increasing the production of eicosapentaenoic acid (EPA) in *Nannochloropsis oculata* (Chaturvedi and Fujita 2006) and to increase the growth properties of Chlorella (Ong et al. 2010) through random mutagenesis.

Typical physical mutagens include different types of irradiation such as UV, gamma or heavy ion beams (Neupert et al. 2009; Hlavová et al. 2015). Among these, mutagenesis using UV light is simple and efficient, since it requires neither specialized equipment nor chemicals and can be performed by merely exposing the cells to germicidal UV lamps. This method has been used extensively in algal

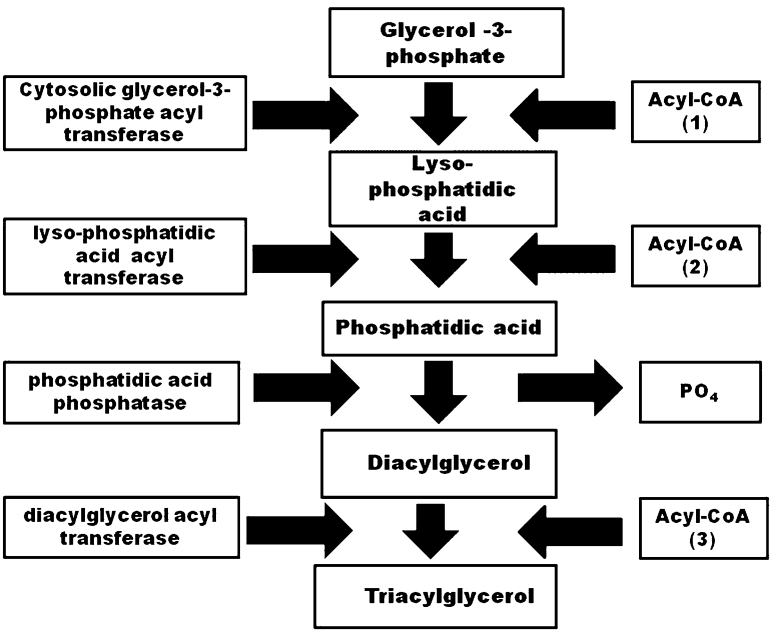

Fig. 10.2 Triacylglycerol biosynthesis pathway in microalgae

research to develop strains with specific features (Neupert et al. 2009) including enhanced oil production (Vigeolas et al. 2012; Jaeger et al. 2014). The applicability of gamma irradiation in microalgae was demonstrated by improved astaxanthin production (Najafi et al. 2011). However, gamma and heavy ion beam irradiation methods require specific equipment, hence not so widely employed in basic research. Though all mutagens have proven their merits in the production of mutants with desired phenotypes through forward genetics, there are some setbacks to this approach (Neupert et al. 2009; Hlavová et al. 2015). Though the initial step of mutagenesis appears simple as there is no need for prior knowledge on the genes of interest, the success of mutation lies in the proper selection of mutants with desired phenotypes (Fig. 10.3). Thousands of mutants may have to be screened to get a single desired phenotype which is one of the major bottlenecks in forward genetic approaches.

Insertional mutagenesis for gene inactivation is an alternative approach to obtain a mutant population for gene identification in various microalgal species (Dent et al. 2005). This can be classified both as forward and reverse genetics due to the nature of the approach. In this method, the mutation of DNA is achieved by insertion of one or two nucleotide bases into the coding region of the gene of interest, which in the loss of expression of that gene (Fang et al. 2006; González-Ballester et al. 2011; Hlavová et al. 2015). The main advantage of insertional mutagenesis over random mutagenesis is the ease of mutant isolation and identification of insertion points on the DNA fragment using specific markers (antibiotic resistant genes or amino acid synthesis gene or T-DNA) (Gonzalez-Ballester et al. 2005).

Table 10.1 Chemical and physical mutagens employed in reverse genetics and their mode of action

Mutagen	Mode of action	Mutation caused
EMS, NTG	Alkylation of DNA base particularly guanine	Point mutations
UV irradiation	Photochemical reaction leading to cyclobutane ring	Point mutations, deletions
Gamma irradiations	Ionization leading to double stranded break	Deletions
Heavy ion beams	Ionization leading to double stranded break	Chromosome breaks and exchanges

Adapted from Hlavová et al. (2015)

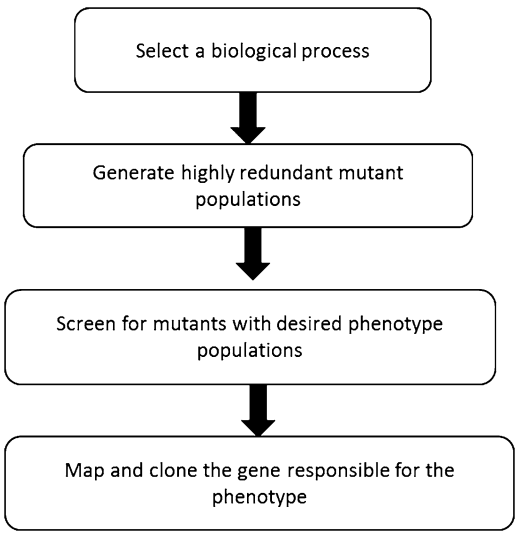

Fig. 10.3 Steps involved in forward genetic approaches (Adapted from Vuttipongchaikij 2012)

4.2 Reverse Genetic Approaches

In contrast to forward genetics, the reverse genetics start with the knowledge on the sequence of genes of interest and aims to alter its natural expression (Fig. 10.4). Overexpression, gene silencing, heterologous expression, homologous recombination, etc. are some of the common methods employed in reverse genetics (Fig. 10.5). A variety of transformation methods have been employed for the successful transformation of foreign DNA into microalgal cells, viz. use of glass beads or silicon carbide whiskers, electroporation, biolistic microparticle bombardment and *Agrobacterium tumefaciens*-mediated gene transfer (Radakovits et al. 2010). Nuclear transformation of microalgae generally results in the random insertion of genes into the host DNA. This may be suitable for transgene expression using random mutagenesis, however, not suitable to delete specific target genes. The screening of transgenic organisms is performed by the use of

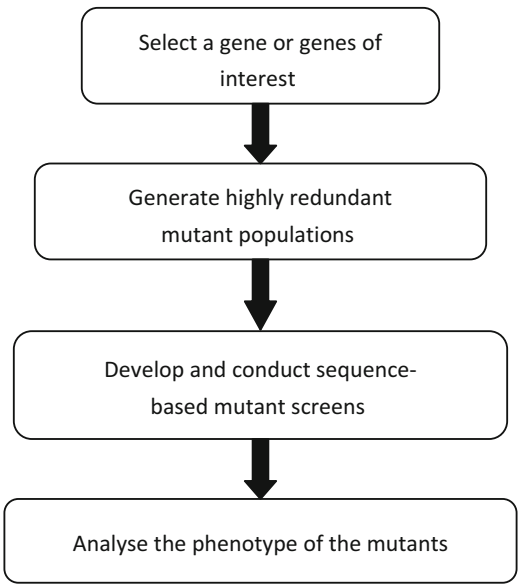

Fig. 10.4 Basic steps involved in reverse genetics (Adapted from Vuttipongchaikij 2012)

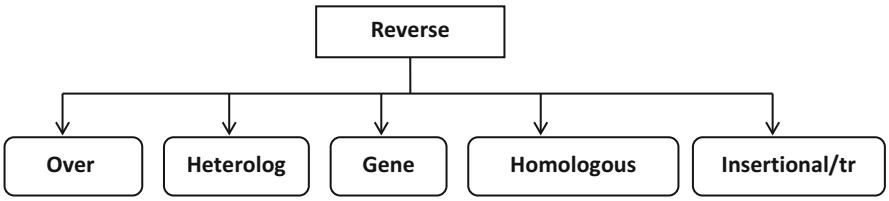

Fig. 10.5 Different reverse genetic approaches that are used for strain improvement in different species of microalgae

markers such as antibiotic resistant genes and fluorescent/biochemical markers (Radakovits et al. 2010). Knockdown of gene expression in *C. Reinhardtii* has been achieved using homologous recombination, however, with low efficiency (Radakovits et al. 2010). RNA silencing has also been successfully employed to knock down gene expression in *C. reinhardtii* and *P. tricornutum* (Radakovits et al. 2010; Kim et al. 2014). Recent improvements in gene silencing strategies include RNA interference (RNAi) or artificial RNA (amiRNA) constructs (Hlavová et al. 2015) or the creation of deletion mutants, either by homologous recombination (Fujiwara et al. 2013) or insertional mutagenesis (Zhang and Hu 2014).

The overexpression of genes is usually achieved by expressing the desired gene with a strong promoter (Gimpel et al. 2013; Hlavová et al. 2015). However, the major limitation of this method is the unpredicted mutation rates that could result due to the insertion of a piece of DNA into the genomic locus. This may sometimes cause additional phenotypic changes unrelated to the expression of the inserted construct. Recent developments in genetic engineering such as gene editing, however, have helped to overcome these limitations. Using these methods, it is possible to make precise changes in the target gene without affecting the expression of other loci. The methods of gene editing have been developed based on the combination of a number of factors which include DNA binding protein and zinc-finger nuclease (ZFN), transcription activator-like effectors (TALEs), gene deletion, gene replacement, introduction of a specific mutation in a gene, and creation of specific gene variants (Boch et al. 2009; Hartung and Schiemann 2014; Puchta and Fauser 2013; Yagi et al. 2014; Hlavová et al. 2015). However, thus far, there are only few reports on microalgae describing the use of ZFN-mediated gene editing (Sizova et al. 2013) and the use of TALEs (Daboussi et al. 2014).

4.3 Advancement in GE Approaches to Improve Lipid Synthesis in Microalgae

Traditionally, GE trails in microalgae have been carried out based on the genetic information on plant genomes. In the last 5 years, substantial progress has been made in microalgal genome research, which would facilitate a better application of GE approaches for strain improvement. Several microalgal nuclear and chloroplast genome projects have been completed thus far which includes: *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Cyanidioschyzon merolae*, *Ostreococcus lucimarinus*, *O. tauri*, *Micromonas pusilla*, *Nannochloropsis* spp., *Lobosphaera (Parietochloris) incisa*, *Trebouxiophyceae* sp., *Chlorella protothecoides*, *C. variabilis*, *C. vulgaris*, *Dunaliella salina*, *Volvox carteri* and *Botryococcus braunii* (Radakovits et al. 2010). The availability of full information on functionally important genes would enable researchers in enhancing production of high-value products from specific microalgal strains in the near future.

Successful genetic transformation has been reported in many species including green (*Chlorophyta*), red (*Rhodophyta*) and brown (*Phaeophyta*) algae; diatoms; euglenids; and dinoflagellates (Radakovits et al. 2010). Members of green algae that have been successfully transformed include *Chlorella reinhardtii*, *C. ellipsoidea*, *C. saccharophila*, *C. vulgaris*, *Haematococcus pluvialis*, *V. carteri*, *C. sorokiniana*, *C. kessleri*, *Ulva lactuca*, *Dunaliella viridis* and *D. Salina*. In most cases, transformation has resulted in stable gene expression. The studies have also indicated that the stability of expression can be improved through proper codon usage or by the use of strong endogenous promoters (Eichler-Stahlberg, et al. 2009; Radakovits et al. 2010). Moreover, the efficiency of transformation is reportedly species dependent and needs to be carefully optimized for each species. Several antibiotic resistance genes have also been optimized for specific microalgal transformant selection. This includes bleomycin, spectinomycin, streptomycin, paromomycin, nourseothricin, hygromycin and chloramphenicol (Radakovits et al. 2010).

Based on the nucleotide sequence knowledge of functional genes in microalgae, numerous trials have been carried out to investigate the feasibility of manipulating the genes of key enzymes relevant to lipid synthesis and to enhance lipid production of different species (Courchesne et al. 2009). Acetyl-CoA (ACCase) carboxylase, the enzyme that catalyses the first committing step of lipid biosynthesis pathway, is one of the commonly exploited enzymes. ACCase is responsible for regulating the rate of de novo fatty acid (FA) biosynthesis in plants and green algae. The modulation of carbon flux is started by the formation of malonyl-CoA through ACCase's activity, and this is the first rate-limiting step in the FA biosynthesis pathway (Davis et al. 2000). ACCase has been purified and characterized in several plants

which include maize (Nikolau and Hawke 1984), parsley (Egin-Bühler and Ebel 1983), spinach (Kannangara and Stumpf 1972) and wheat (Egin-Bühler et al. 1980) and also in microalgae (Livne and Sukenik 1990; Roessler 1990). Overexpression of this enzyme reportedly improved lipid accumulation in few species (Courchesne et al. 2009; Blatti et al. 2013; Liang and Jiang 2013). Davis et al. (2000) observed a sixfold increase in fatty acid synthesis rate while co-expressing *E. coli* ACCase (encoded by accA, accB, accC, accD) and thioesterases I (encoded by the tesA gene). These results confirmed that ACCase catalyzing the committing step was unquestionably the rate-limiting step for the fatty acid biosynthesis in *E. coli*. Overexpression of ACCase genes in *Brassica napus* improved lipid accumulation by 5 % (Thelen and Ohlrogge 2002). The microalgal ACCase was first isolated by Dunahay et al. (1996) from *Cyclotella cryptica* and successfully expressed in *Cyclotella cryptica* and *Navicula saprophila*. Though activity of this enzyme was increased in transgenic microalgal strains, there was no increase in lipid accumulation, which could be due to ACCase activity not being the rate-limiting step for lipid biosynthesis in these species (Dunahay et al. 1996).

Acyl-CoA:diacylglycerol acyltransferase (DGAT) is the most important enzyme in the Kennedy pathway (TAG biosynthesis) which converts diacylglycerol to triacylglycerols (Liang and Jiang 2013). The expression level of this gene has been checked in many plants such as *Brassica napus*, *Arabidopsis thalia*na and *Nicotiana tabacum*. Acyl-CoA:diacylglycerol acyltransferase gene from *Arabidopsis* was transformed into yeasts and tobacco, and DGAT activity as well as TAG accumulation was analysed. In transgenic yeasts, 200–600-fold increase in DGAT activity was observed, whereas TAG accumulation was only increased by three- to ninefold (Thelen and Ohlrogge 2002; Liang and Jiang 2013). Thus far only few studies have been reported for DGAT expression in microalgae. Wagner et al. (2010) have identified and characterized acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) genes from the microalga, *Ostreococcus tauri.*

La Russa et al. (2012) overexpressed DGAT2 gene in the microalgae *Chlamydomonas reinhardtii* and observed an increase in (1.7–29.1 times) mRNA level as compared to wild strain. However, the increased level of mRNA expression did not result in elevated accumulation of TAG. This indicates the need for a comprehensive research in other microalgal strains to identify the role of DGAT and to develop strategies to enhance lipid accumulation (Yu et al. 2011). Other enzymes that may be targeted for enhanced fatty acid and TAG synthesis in microalgae are fatty acid synthetase (FAS), lysophosphatidate acyltransferase (LPAT), acetyl-CoA synthase (ACS), malic enzyme (ME) and ATP citrate lyase (ACL) (Liang and Jiang 2013). These enzymes are extensively studied in plants for improving lipid yields.

Apart from the above enzymes, overexpression of malic enzyme (ME), an enzyme that plays a major role in lipid biosynthesis by conversion of malate to pyruvate, is also reported to increase lipid biosynthesis in plants and microalgae (Zhang et al. 2007; Courchesne et al. 2009; Li et al. 2013; Xue et al. 2013). Recently, Xue et al. (2015) reported that an overexpression of ME gene (PtME) significantly enhanced the enzymatic activity of transgenic *Phaeodactylum tricornutum*. In their study, a 2.5-fold increase in total lipid content in the transgenic cells was observed with a similar growth rate to wild type. Further, an increase in the neutral lipid content to 31 % was also noticed under nitrogen-deprivation conditions on this transgenic *P. tricornutum*.

Multiple gene expression, i.e. overexpression of more than one key enzyme in the TAG pathway to enhance lipid biosynthesis, was also suggested by few researchers (Verwoert et al. 1995; Roesler et al. 1997; Li et al. 2013). Overexpression of the three functional genes, viz. acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA:lysophosphatidic acyltransferase (LPAAT) and acyl-coA:diacylglycerol acyltransferase (DGAT), enhanced the quantity (up to twofold) of storage lipid in *C. minutissima* UTEX 2219 (Hsieh et al. 2012). Recently, Talebi et al. (2014) have successfully accomplished manipulation of carbon flux into fatty acid biosynthesis pathway in *Dunaliella salina*. This was achieved by transferring the pGH vector harbouring ME and AccD genes into the chloroplast genome of *D. Salina* using particle bombardment. The comparison of lipid and fatty acid profile of the transformed algal cell lines and control revealed a stable and overexpression of ME/AccD genes in the transformants, leading to a 12 % increase in total lipid content and substantial improvement in biodiesel properties.

Suppression of gene expression in the comparative pathways is another method to enhance lipid production in microalgae. Carbohydrate metabolism is the most important pathway to store and accumulate carbon as starch in many microalgae (Gonzalez-Fernandez and Ballesteros 2012; Ho et al. 2013). Inhibiting the carbohydrate metabolism may result in carbon flow towards lipid biosynthesis. A *Chlamydomonas* mutant has shown tenfold increase in the TAG accumulation after deactivation of ADP-glucose pyrophosphorylase, which catalyses the committed step in starch metabolism (Li et al. 2010). These findings indicated the probability of an increase in lipid production by redirecting the carbon from starch synthesis to lipid accumulation. However, knockdown of starch synthesis may result in decreased growth, thereby affecting biomass and lipid productivity.

Suppression of lipid catabolism is another potential strategy to be employed in microalgae to increase lipid accumulation. A mutant strain of *Thalassiosira pseudonana* has shown 3.5-fold higher lipid content after alteration in the

lipid catabolism by knocking down the regulation of multifunctional enzymes, lipase/phospholipase/acyltransferase (Trentacoste et al. 2013). The genes regulating these enzymes can be targeted for high lipid accumulation, without compromising growth. The quality of microalgal lipids however dictates the properties of subsequently produced biodiesel (Blatti et al. 2013). Therefore, a need exists to develop new strategies to improve the quality of microalgal lipids for the biodiesel to meet standard specifications.

The most suitable fatty acids for biodiesel production are saturated and monounsaturated fatty acids, preferably with a carbon length, 12:0, 14:0, 16:0, 16:1, 18:0 and 18:1 (Blatti et al. 2013). An enzyme, acyl-ACP thioesterase, regulates the fatty acid chain length by releasing the fatty acid chain from fatty acid synthase (Guo et al. 2014). Thioesterases from different organisms are specific for a particular chain length of fatty acids as per the metabolic needs of that organism. If the thioesterase genes from various organisms responsible for different chain lengths of fatty acids are transformed into microalgae, it could greatly improve the fatty acid composition desired for biodiesel synthesis. Two shorter chain length fatty acid acyl-ACP thioesterases from *Cinnamomum camphora* and *Umbellularia californica* were transformed to microalgae *Phaeodactylum tricornutum* (Radakovits et al. 2011). The fatty acid profile of transgenic *P. tricornutum* showed an increase in percentage composition of lauric (C12:0) and myristic (C14:0) acids (Xue et al. 2015). These molecular strategies for improving microalgal lipid quality hold immense significance for the successful production of microalgal biodiesel with the required specifications.

4.4 Bottlenecks in Microalgal GE Approaches and the Way Forward

GE approaches in microalgae have tremendous scope in enhancing lipid production; however, there are major limitations during scaleup of production. Though the commercial application of algal transgenics is beginning to be realistic due to the advancements in molecular techniques, there are challenges that are still needed to be overcome to use transgenic microalgae as a powerful tool for the production of commercial biomolecules (Cadoret et al. 2008). One of the major challenges in GE is the screening process, which is a critical parameter for both forward and reverse genetic approach (Hlavová et al. 2015). With the traditional methods, thousands of mutants may have to be screened to get the desired phenotype. However, various strategies have been proposed to improve the screening process which includes methods based on the survival of either mutant or wild type cells, under defined conditions. It is equally important to maintain similar screening conditions for all mutants in order to compare them directly, which is practically challenging (Hlavová et al. 2015).

Another key challenge is instability or loss of gene expression as the mutants are prone to reversion. The probability of reversion and stable expression, however, depends on many factors. It is reported that the large fragment of foreign DNA inserted into the genome by insertional mutagenesis is prone to reversion by gene silencing (Hlavová et al. 2015). This also could have a larger impact on the mutant strain, since silencing might spread to neighbouring regions of the genome. This could be partially overcome by growing the mutant strain constantly in the presence of antibiotics, if the antibiotic resistant genes were used as markers (Neupert et al. 2009; Hlavová et al. 2015). It is also recommended to limit subculturing by storing the mutant strain in liquid nitrogen to avoid mutant reversion. In the case of reverse genetics, the probability of gene reversion increases with the expression level, which could be partially avoided if an inducible promoter is used during the transcript construction (Iwai et al. 2014; Hlavová et al. 2015).

Possibility of low or no expression due to the absence of specific codons is another challenge that could affect the transgenic microalgae. It has been demonstrated that microalgal genomes contain very high GC content, for example, 71 % in *Monoraphidium* and 61 % in *Chlamydomonas* (Jarvis et al. 1992). This suggests a high variation in codon usage in microalgae as compared to other organisms. Therefore, codon optimization may be a prerequisite to improve the gene expression in different microalgal species. Codon optimization in *Chlamydomonas* showed a fivefold increase in its expression level (Fuhrmann et al. 1999; Hlavová et al. 2015). With an increase in the number of genome sequences being available for different microalgal species, researchers would be able to overcome this limitation in the near future.

Another major challenge is the suitability of transgenic microalgae for large-scale cultivation. Upon exposure to large-scale cultivation conditions (large-scale ponds), the strains experience situations that are more diverse from the controlled lab conditions, which subsequently affect its transgenic property. Consequently, productivity in outdoor cultivation never reaches that of the optimized laboratory conditions. Performances in large-scale ponds are sometimes also dependent on the mutant stability (Hlavová et al. 2015). Another major bottleneck in microalgal biotechnology is the cost involved in the whole transgenic process. Reverse genetics requires highly specialized equipment, and some processes are time consuming due to the numerous steps involved, from screening to large-scale cultivation, which would affect the cost immensely. Moreover, some countries do not support transgenics due to legislative policies against GMOs.

5 Conclusions

In microalgal research, considerable interest has been placed on bioprospecting, which is one of the key elements exercised for strain improvement. Various cultivation techniques are also used in practice for improving microalgal lipid biosynthesis but, however, face challenges during its scaleup. With the current pace in biotechnology advancements, genetic engineering in microalgae would be the most appropriate, efficient and cost-effective method for strain improvement. Both forward and reverse genetics have been employed in microalgal strain improvements. The forward genetics using chemical and physical mutagens is relatively simple; however, the screening process is extensive or exhaustive. In reverse genetics, specific knowledge on the target gene is imperative and initial optimization of each step is time consuming and costly. The genome sequencing of many microalgal species has been completed, and this would definitely form a strong foundation for improving the GE approaches for increased lipid production in the near future.

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