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Review

Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches

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ABSTRACT

This paper compares three possible strategies for enhanced lipid overproduction in microalgae: the biochemical engineering (BE) approaches, the genetic engineering (GE) approaches, and the transcription factor engineering (TFE) approaches. The BE strategy relies on creating a physiological stress such as nutrient-starvation or high salinity to channel metabolic fluxes to lipid accumulation. The GE strategy exploits our understanding to the lipid metabolic pathway, especially the rate-limiting enzymes, to create a channelling of metabolites to lipid biosynthesis by overexpressing one or more key enzymes in recombinant microalgal strains. The TFE strategy is an emerging technology aiming at enhancing the production of a particular metabolite by means of overexpressing TFs regulating the metabolic pathways involved in the accumulation of target metabolites. Currently, BE approaches are the most established in microalgal lipid production. The TFE is a very promising strategy because it may avoid the inhibitive effects of the BE approaches and the limitation of "secondary bottlenecks" as commonly observed in the GE approaches. However, it is still a novel concept to be investigated systematically.

Contents

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1. Introduction

Biodiesel is one of the most promising renewable transportation fuels that have achieved remarkable success worldwide. According to a World Bank report (2008), 6.5 billion litres of biodiesel was produced worldwide in 2006, 75% of which by the European Union and 13% by the USA. The current contribution of biodiesel to global transportation fuel consumption is, however, only 0.14% and the favourable policies of major countries in the world are expected to increase this contribution by 5 times by 2020. It is therefore predictable that massive global demand on renewable energy will continue to drive the rapid growth of biodiesel production in an unprecedented scale. Nevertheless, current increase of food prices worldwide had brought about public awareness and concerns regarding the competition for agricultural resources between the food industry and the energy sector. Development of sustainable and cost-effective alternatives to the traditional agricultural and forestry crops is therefore of urgent need for sustainable biofuel production.

Oil-rich microalgae have been demonstrated to be a promising alternative source of lipids for biodiesel production (Chisti, 2007; Li et al., 2008b; Song et al., 2008; Walker et al., 2005b; Wang et al., 2008). There seems to be little doubt that fast growing microalgae should be able to provide enough renewable biofuels for the replacement of fossil transportation fuels (Li et al., 2008b). An integrated strategy was proposed to enhance the economical cost-effectiveness and environmental sustainability by combining the benefits of biofuel production, CO_2 mitigation, waste heat utilization, wastewater treatment and novel bioproduct production using the microalgal cultivation processes (Li et al., 2008b; Wang et al., 2008). Nevertheless, significant challenges remain in the economics of microalgal biodiesel production and extensive studies have been carried out to cope with these challenges. In this minireview, we focus on the progress, challenges, and future perspectives of lipid overproduction using microalgae by different approaches, including the BE, GE, and the emerging TFE approaches.

2. Biochemical engineering approaches

The BE approach here refers to the strategy of enhancing lipid production of microalgae by controlling the nutritional or cultivation conditions (e.g., temperature, pH, and salinity) to channel metabolic flux generated in photobiosynthesis into lipid biosynthesis. Nutrient-starvation has so far been the most commonly employed approach for directing metabolic fluxes to lipid biosynthesis of microalgae. In this scenario, microalgae accumulate lipids as a means of storage under nutrient limitation when energy source (i.e., light) and carbon source (i.e., CO_2) are abundantly available and when the cellular mechanisms for the photobiosynthesis are active. While a number of nutrients such as phosphorus and iron deficiency have been reported as being able to cause cell growth cessation and channel metabolic flux to lipid/fatty acid biosynthesis, nitrogen is the most commonly reported nutritional limiting factor triggering lipid accumulation in microalgae.

Nitrogen-starvation has been observed to lead to lipid accumulation in a number of microalgal species. For instance, *Chlorella* usually accumulates starch as storage material. However, it was observed by Illman et al. (2000) that *C. emersonii*, *C. minutissima*, *C. vulgaris*, and *C. pyrenoidosa* could accumulate lipids of up to 63%, 57%, 40%, and 23% of their cells on a dry weight basis, respectively, in low-N medium. *Neochloris oleoabundans* was reported, under nitrogen deficient conditions, to be able to accumulate 35–54% lipids of its cell dry weight and its TAGs comprised 80% of the total lipids (Kawata et al., 1998; Tornabene et al., 1983). It was also observed (Yamaberi et al., 1998) that the TAGs accumulated in *Nannochloris* sp. cells could be 2.2 times as that in the cells in nitrogen sufficient cultures. Our studies (Li et al., 2008a) showed that sodium nitrate was the most favourable nitrogen source for cell growth and lipid production of *N. oleoabundans* among the three tested nitrogen-containing compounds, i.e., sodium nitrate, urea, and ammonium bicarbonate. It was observed that lipid cell contents decreased with the increase of sodium nitrate in the medium in the range of 3–20 mM. The trend that lower nitrogen source concentration in medium led to higher lipid cell content was hypothetically explained by the fact that nitrogen would have exhausted earlier at low cell density when the initial concentration of nitrogen source in medium was low. As a result, cells started to accumulate lipid when light had good penetration (at low cell density), when individual cells were exposed to a large quantity of light energy, resulting in more metabolic flux generated from photosynthesis to be channelled to lipid accumulation on an unit biomass basis.

Phosphate limitation was also observed to cause enhancement of lipid accumulation of *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006). With decreasing phosphate availability from 175 to 52.5, 17.5 and 0 μM (K_2HPO_4), the cellular total lipid content of starved cells increased, mainly due to the drastic increase in TAG levels. In the absence of phosphate, the proportion of phospholipids was reduced from 8.3% to 1.4% of total lipids, and the proportion of TAG increased from 6.5% up to 39.3% of total lipids. Furthermore, iron deficiency has also been reported to stimulate lipid accumulation in microalgae *Chlorella vulgaris*, which accumulated up to 56.6% lipid of biomass by dry weight under the optimal condition (1.2×10^{-5} mol $FeCl_3$) (Liu et al., 2008).

In addition to nutrient-starvation, other stress conditions may also cause enhanced accumulation of lipids in microalgae. For instance, Takagi et al. (2006) observed that TAG content increased in *Dunaliella*, a marine alga, under high salinity conditions. In that research, an initial NaCl concentration higher than 1.5 M was found to markedly inhibit cell growth. However, when the initial NaCl concentration increased from 0.5 (equal to seawater) to 1.0 M, it resulted in a higher intracellular lipid content (67%) in comparison with 60% for the salt concentration of 0.5 M. Addition of 0.5 or 1.0 M NaCl at mid-log phase or the end of log phase during cultivation with initial NaCl concentration of 1.0 M further increased the lipid content to 70%.

An inherited disadvantage of the BE strategy is, however, nutrient-starvation or the physiological stress required for accumulating high lipid content in cells is associated with reduced cell division (Ratledge, 2002). Since lipids are intracellular products, the overall lipid productivity is the product of cell lipid content multiplied by biomass productivity. The overall lipid/energy productivity will therefore be compromised due to the lowered biomass productivity. For instance, Scragg et al. (2002) studied the energy recovery

of *C. vulgaris* and *C. emersonii* grown in Watanabe's medium and a low nitrogen medium. The results showed that the low nitrogen medium, although induced higher lipid accumulation in both algae with high calorific values, the overall energy recovery was lower with the low nitrogen medium than that with the Watanabe's medium. Our studies (Li et al., 2008a) also showed that, in the tested range of 3–20 mM sodium nitrate, although the highest cell lipid content of 40% was obtained at the lowest sodium nitrate concentration of 3 mM, the maximal lipid productivity was achieved at 5 mM.

A commonly suggested countermeasure is to use a two-stage cultivation strategy, dedicating the first stage for cell growth/division in nutrient-sufficient medium and the second stage for lipid accumulation under nutrient-starvation or other physiological stress. Indeed, a well formulated medium as the one proposed by our group in a previous study (Li et al., 2008a) would achieve the two-stage lipid production "naturally" as the cells will be able to grow quickly before the exhaustion of the limiting substrate (N in this particular case) and then switch to lipid accumulation under N-starvation conditions. Furthermore, a hybrid closed photobioreactor/open pond microalgal cultivation system (Huntley and Redalje, 2007) was suggested to be potentially the appropriate engineering solution accommodating the two-stage strategy with the photobioreactors dedicated to nutrient-rich inoculum build-up and the open ponds to low-nutrient lipid accumulation (Schenk et al., 2008). It was also pointed out that employment of low-nutrient media in open ponds is not only beneficial for lipid accumulation and contamination control, but also environmentally friendly.

Nevertheless, deficiency of these nutrients may slow down photosynthesis of microalgal cells one way or the other, resulting in lowered overall lipid productivity. Many of the commonly used limiting nutrients are essential for photosynthesis of microalgae and the depletion of which may severely impede the photosynthesis responsible for generating the metabolic flux for lipid production. For instance, it was observed in our studies that chlorophyll, the essential pigment for light capturing in the biosynthesis of green alga *N. oleoabundans*, was consumed for cell growth when nitrogen was exhausted from the medium, resulting in a sharp drop of chlorophyll cell content (Li et al., 2008a). Phosphorus is essential to the cellular processes related to energy bio-conversion (e.g., photophosphorylation). Of particular relevance, photosynthesis requires large amounts of proteins (notably Rubisco) and proteins are synthesized by phosphorus-rich ribosomes (Wang et al., 2008). As a result, channelling metabolic flux to lipid biosynthesis by the means of phosphate starvation may have a severe impact on photosynthesis.

There is apparently a dilemma in the BE strategy, i.e., the very reason that stimulates lipid accumulation in cells may result in severely impeded cell growth and photosynthesis and hence lowered overall lipid productivity. This dilemma could likely be solved by employing metabolic engineering approaches aiming at enhancing the metabolic flux into lipid biosynthesis without applying the aforementioned "artificial" physiological stresses.

3. The genetic engineering approaches

Although biotechnological processes based on transgenic microalgae are still in their infancy, researchers and companies are considering the potential of microalgae as green cell-factories to produce value-added metabolites and heterologous proteins for pharmaceutical applications (Leon-Banares et al., 2004). The commercial application of algal transgenics is beginning to be realized and algal biotechnology companies are being established. It was predicted that microalgae, due to the numerous advantages they present, could offer a powerful tool for the production of commercial molecules in a near future (Cadoret et al., 2008).

The fast growing interests in the use of transgenic microalgae for industrial applications is powered by the rapid developments in microalgal biotechnology. Complete genome sequences from the red alga *Cyanidioschyzon merolae* (Nozaki et al., 2007), the diatoms *Thalassiosira pseudonana* (Armbrust et al., 2004) and *Phaeodactylum tricornutum* (Bowler et al., 2008) and the unicellular green alga *Ostreococcus tauri* (Derelle et al., 2006) have been completed. Nuclear transformation of various microalgal species is now a routine, chloroplast transformation has been achieved for green, red, and euglenoid algae, and further success in organelle transformation is likely as the number of sequenced plastid, mitochondrial, and nucleomorph genomes continues to grow (Walker et al., 2005a). Various genetic transformation systems have been developed in green algae such as *Chlamydomonas reinhardtti* and *Volvox carteri* (Walker et al., 2005a).

The fast developments of microalgal biotechnology permit the isolation and use of key genes for genetic transformation. Of particular relevance, acetyl-CoA carboxylase (ACC) was first isolated from the microalga *Cyclotella cryptica* in 1990 by Roessler (1990) and then successfully transformed by Dunahay et al. (Dunahay et al., 1995, 1996; Sheehan et al., 1998) into the diatoms *C. cryptica* and *Navicula saprophila*. The ACC gene, *acc*1, was overexpressed with the enzyme activity enhanced to 2–3-folds. These experiments demonstrated that ACC could be transformed efficiently into microalgae although no significant increase of lipid accumulation was observed in the transgenic diatoms (Dunahay et al., 1995, 1996). It also suggests that overexpression of ACC enzyme alone might not be sufficient to enhance the whole lipid biosynthesis pathway (Sheehan et al., 1998).

Even though there is no success story with respect to lipid overproduction of microalgae using the GE approach up to now, a solid understanding towards the global TAG biosynthesis pathway, which is generally accepted to be identical throughout all species except the differences in the location of reactions and the structure of some key enzymes, has been established. Extensive studies have also been carried out regarding the enhancement of lipid production using the GE approach in different species. These results provide a valuable background for future studies with microalgae.

3.1. An overview of the global lipid biosynthesis pathway

As shown in Fig. 1, the global synthesis pathway of TAG in cells is comprised of three major steps: (1) carboxylation of acetyl-CoA to form malonyl-CoA, the committing step of fatty acid biosynthesis; (2) acyl chain elongation; and (3) TAG formation. The enzymes involved in each step of the pathway and their functionalities are discussed briefly as follows.

3.1.1. The committing step

As shown in Fig. 1, lipid biosynthesis starts with the acetyl-CoA carboxylase (ACC), which catalyzes the important committing step of the fatty acid synthetic pathway, the biotin-dependant carboxylation of acetyl-CoA to form malonyl-CoA (Davis et al., 2000; Kim, 1997; Li and Cronan, 1993; Sendl et al., 1992). In *Escherichia coli*, ACC is a protein containing four subunits, which are encoded by genes accA, accB, accC and accD that are located at different positions on the chromosome (Li and Cronan, 1993). It is a trifunctional enzyme with a biotin carboxyl carrier protein, a biotin-carboxylase subunit and a carboxyl-transferase subunit (Sendl et al., 1992) joined together into a heterotrimeric complex (Tehlivets et al., 2007). In contrast, eukaryotic cells encode a multi-domain single polypeptide, which is responsible for all the functions of the ACC (Sasaki and Nagano, 2004; Tehlivets et al., 2007). In animal cells, ACC is located in the cytoplasm and thus has to use cytosolic acetyl-CoA for malonyl-CoA formation and acyl chain elongation. Yeasts have

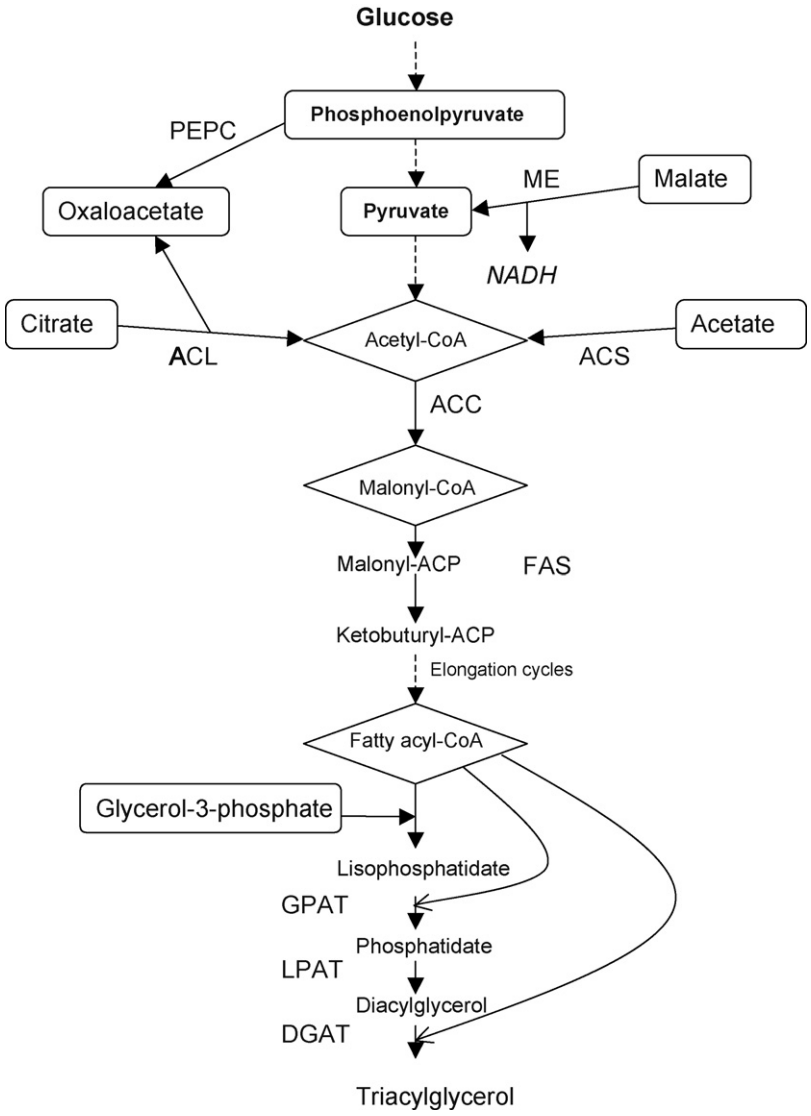

Fig. 1. The fatty acid and TAG biosynthesis.

both cytosolic and mitochondrial ACC, but it has been demonstrated to be able to survive with a non-functional mitochondrial enzyme (Tehlivets et al., 2007). In plants, fatty acid synthesis occurs entirely in plastids of developing seeds, and ACC uses the acetyl-CoA that is found in this organelle (Dyer and Mullen, 2005; Roesler et al., 1997). The plastid ACC has a different structure than the cytosolic ACC. It is a multi-subunit prokaryotic type enzyme, as opposed to the multifunctional eukaryotic type located in the cytoplasm.

3.1.2. Acyl chain elongation

Once malonyl-CoA is synthesized, it is transferred by malonyl-CoA:ACP transacetylase to the acyl-carrier protein (ACP) of the fatty acid synthase (FAS) multi-enzymatic complex (Subrahmanyam and Cronan, 1998). Bacteria and plants have type II FAS (Rock and Jackowski, 2002), which is a multi-subunit protein in which each individual peptide is dissociable and can catalyze an enzymatic reaction, as opposed to the type I FAS found in yeast and vertebrates, which is a multifunctional protein (Verwoert et al., 1995).

FAS catalyzes fatty acid elongation by condensing malonyl-CoA molecules and acetyl-CoA. ACP, one of the FAS subunits, contains a thiol group that can form malonyl-ACP via forming thioesters with malonyl-CoA, and afterwards with the growing acyl chain in order to assure its transport (Subrahmanyam and Cronan, 1998). ACP can also fix acetyl by forming acetyl-ACP. Then, the acetyl-group is transferred to another subunit of the FAS, the ketoacyl-ACP synthase (KAS), which catalyzes the condensation of malonyl-ACP or the growing acyl chain to form ketobutyryl-ACP or ketoacetyl-ACP. This resulting compound is first transformed via three successive reactions, i.e., reduction, dehydration and reduction, and then condensed with another malonyl-CoA. This cycle is repeated until the saturated chain of a palmitic (16:0) or a stearic acid (18:0) is formed (Subrahmanyam and Cronan, 1998). At last, ACP-thioesterase cleaves the acyl chain and liberates the fatty acid.

To obtain longer or unsaturated chains, elongases and desaturases are required, which act on palmitate or stearate. These enzymes are located in endoplasmic reticulum membrane and mitochondria. They can produce long chain fatty acids, as well as unsaturated acyl chains. They then act on the composition of the fatty acid pool but not on their accumulation level. Many experiments have been carried out to modify the lipid content in transgenic plants using these enzymes, such as the increase of omega-3 production (Budziszewski et al., 1996; Dehesh, 2001; Graham et al., 2007; Ivy et al., 1998; Napier, 2007; Napier et al., 2004; Opsahl-Ferstad et al., 2003; Stoll et al., 2005; Zou et al., 1997).

Table 1
Lipid synthesis enhancement genes (enzymes).

Gene (enzyme)	Source-species	Receiver-species	Note	Refs
accA, accB, accC, accD, (ACC), tesA (thioesterase I)	*E. coli* (BL21) (bacteria)	*E. coli* (BL21) (bacteria)	6× fatty acid synthesis	Davis et al. (2000)
Acc1 (cytosolic ACC)	*Arabidopsis* (plant)	*Brassica napus* (plant)	1–2× plastid ACC + 6% fatty acid content	Roesler et al. (1997)
Acc1 (ACC)	*Arabidopsis* (plant)	*Solanum tuberosum* (plant)	5× TAG content	Klaus et al. (2004)
Acc1 (ACC)	*Cyclotella cryptica* (alga)	*Cyclotella cryptica* (alga)	2–3× ACC activity, no change in lipid content	Dunahay et al. (1995) and Dunahay et al. (1996)
Acc1 (ACC)	*Cyclotella cryptica* (alga)	*Navicula saprophila* (alga)	2–3× ACC activity, no change in lipid content	Dunahay et al. (1995) and Dunahay et al. (1996)
fabF (KAS II)	*E. coli* (bacteria)	*E. coli* (bacteria)	Toxic (CoA pool from 0.5–40% in malonyl-CoA)	Subrahmanyam and Cronan (1998)
fabH (KAS III)	*E. coli* (bacteria)	*Brassica napus* (plant)	Stress, arrest of the cell growth	Verwoert et al. (1995)
KAS III	*Spinacia oleracea* (plant)	*Nicotiana tabacum* (plant)	16:0 accumulation lower oil content	Dehesh et al. (2001)
KAS III	*Spinacia oleracea* (plant)	*Arabidopsis* (plant)	16:0 accumulation lower oil content	Dehesh et al. (2001)
KAS III	*Spinacia oleracea* (plant)	*Brassica napus* (plant)	16:0 accumulation lower oil content	Dehesh et al. (2001)
LPAT	*Saccharomyces cerevisiae* (yeast)	*Brassica napus* (plant)	6× oil content	Zou et al. (1997)
are1 and are2 (DGAT)	*Arabidopsis thaliana* (plant)	Yeast	3–9× TAG content	Bouvier-Nave et al. (2000)
are1 and are2 (DGAT)	*Arabidopsis thaliana* (plant)	*Nicotiana tabacum* (plant)	7× TAG content	Bouvier-Nave et al. (2000)
DGAT	*Arabidopsis* (plant)	*Arabidopsis* (plant)	+10–70% oil content	Jako et al. (2001)
acs (ACS)	*E. coli* (MG1655) (bacteria)	*E. coli* (MG1655) (bacteria)	9× ACS activity, increased acetate assimilation	Lin et al. (2006)
malEMt and malEMc (ME)	*Mortierella alpina* and *Mucor circinelloides* (fungi)	*Mucor circinelloides* (fungi)	2.5× lipid accumulation	Zhang et al. (2007)
ACL	Rat	Tobacco	+16% lipid content	Rangasamy and Ratledge (2000)
Antisens PEP gene	*Agrobacterium tumefaciens*	*Brassica napus*	+6.4–18% oil content	Chen et al. (1999)

3.1.3. Triacylglycerol (TAG) formation

For eukaryotes, TAG formation takes place in specialized organelles, i.e., the mitochondria or/and plastid (plants only) located in the endoplasmic reticulum. In contrast, the TAG synthesis takes place in the cytoplasm of prokaryotic cells. This process yields neutral lipids, a way to store fatty acids and thus energy (Rajakumari et al., 2008). Storage of high energy density TAG allows cells to have more free space (Coleman and Lee, 2004).

The first step of TAG synthesis is the condensation (acylation) of glycerol-3-phosphate (G3P) with an acyl-CoA to form lysophosphatidate (LPA), which is catalyzed by acyl-CoA:glycerol-*sn*-3-phosphate acyl-transferase (GPAT). This enzyme exhibits the lowest specific activity of the TAG synthesis pathway, and was suggested to be potentially the rate limiting step (Cao et al., 2006; Coleman and Lee, 2004). It is subjected to many regulatory controls at the transcriptional level, at the post-transcriptional level (e.g., by means of post-transcriptional phosphorylation or dephosphorylation) and by allostery.

The LPA is then further condensed, catalyzed by acyl-CoA:acylglycerol-*sn*-3-phosphate acyl-transferase (GPAT), with another acyl-CoA to produce phosphatidate (PA) (Athenstaedt and Daum, 1999). Afterwards, PA can be dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol.

At last, synthesis of TAG is catalyzed by acyl-CoA:diacylglycerol acyl-transferase (DGAT), which incorporates the third acyl-CoA into the diacylglycerol molecule. This enzyme is also known as an important regulator for this pathway (Oelkers et al., 2002; Sandager et al., 2002). TAGs can then be stored in oil bodies (Murphy, 2001).

3.2. Overexpression of TAG biosynthesis pathway enzymes

Numerous studies have been carried using the GE strategy to enhance the lipid accumulation in different species. Some of these studies have been summarized in Table 1 and will be discussed briefly in this section.

3.2.1. Acetyl-CoA carboxylase (ACC)

Since the successful demonstration by Page et al. (1994) that ACC exerts a strong control on the metabolic flux of fatty acid synthesis in plants, this enzyme has been overexpressed in different species for enhanced lipid production. For instance, the cytosolic ACC from *Arabidopsis* was overexpressed in *Brassica napus* (rapeseed) plastid with a 1–2-fold increase of plastid ACC activity (Roesler et al., 1997). However, the fatty acid content of the recombinant was only 6% higher than the control, suggesting that a "secondary bottleneck", i.e., another limiting step, in the fatty acid synthesis pathway might have emerged as a result of the removal of the primary bottleneck. Davis et al. (2000) cloned the four ACC genes, accA, accB, accC and accD of *E. coli* BL21 and overexpressed them in the same strain. ACC subunits were produced in equimolar quantities. This caused an increase of the intracellular malonyl-CoA pool as a result of the enhanced ACC enzymatic activity. A 6-fold increase in the rate of fatty acid synthesis was observed, confirming that the ACC catalyzed committing step was indeed the rate-limiting step for fatty acid biosynthesis in this strain. However, the lack of lipid production enhancement seemed to suggest again that a secondary limiting-step after fatty acid formation prevented the efficient conversion of fatty acids to lipids in *E. coli*. As mentioned previously, ACC was also isolated from microalgae (Roessler, 1990) and successfully overexpressed in the diatoms *C. cryptica* and *N. saprophila*. Not surprisingly, no significant increase of lipid accumulation was observed in the transgenic diatoms (Dunahay et al., 1995, 1996).

Sheehan et al. (1998) suggested that overexpression of ACC enzyme alone may not be sufficient to enhance the whole lipid biosynthesis pathway in diatoms. This conclusion seems to be generally true for most species because enhanced lipid accumulation was rarely reported even though significant enhancement of relevant enzymes and/or intermediate products such as fatty acids was commonly observed. This is probably due to either or both of the following two reasons: (1) the committing step catalyzed by ACC is not the rate-limiting step in a particular species and (2) a secondary

rate-limiting step, i.e., the "secondary bottleneck", emerged when ACC was overexpressed. Nevertheless, Klaus et al. (2004) achieved an increase in fatty acid synthesis and a more than 5-fold increase in the amount of TAG in *Solanum tuberosum* (potato) by overexpressing the ACC from *Arabidopsis* in the amyloplasts of potato tubers.

3.2.2. Fatty acid synthetase (FAS)

Trials in overexpressing the KAS subunit of FAS in *E. coli* were carried out to facilitate the C2 concatenation. However, this manipulation was found extremely toxic for the cell (Subrahmanyam and Cronan, 1998). In another trial, an *E. coli* KAS III was overexpressed in the rapeseed (Verwoert et al., 1995), which caused a major change in the fatty acid composition profile with the increase of short-chain fatty acids (14:0) and a decrease of 18:1 fatty acids. This modification caused a response to stress, which significantly affected the growth of the plant cells. Similarly, KAS III from spinach *Spinacia oleracea* was overexpressed by Dehesh et al. (2001) in tobacco *Nicotiana tabacum*, cress *Arabidopsis* and rapeseed, resulted in a reduction of the rate of lipid synthesis and an accumulation of 16:0 fatty acids.

It seems that the subunits of FAS are challenging targets for metabolic engineering for fatty acid metabolism enhancement, probably due to the fact that FAS is a multi-enzymatic complex containing subunits whose activities depend on one another. The difficulties experienced with the heterologous expression of multi-enzymatic complexes such as FAS were also likely due to the differences in multipoint controls among different species.

3.2.3. Lysophosphatidate acyl-transferase (LPAT)

Transformation of rapeseed with a putative *sn*-2-acyl-transferase gene from the yeast *Saccharomyces cerevisiae* was carried out by Zou et al. (1997), leading to overexpression of seed lysophosphatidate acyl-transferase (LPAT) activity. This enzyme is involved in TAG formation and its overexpression led to increases from 8% to 48% seed oil content on the seed dry weight basis. However, it was cautioned that the steady-state level of diacylglycerol could be perturbed by an increase of LPAT activity in developing seeds.

3.2.4. Acyl-CoA:diacylglycerol acyl-transferase (DGAT)

Acyl-CoA:diacylglycerol acyl-transferase (DGAT) catalyzes, as discussed previously, the last step of TAG formation to form triacylgycerol from diacylglycerol and fatty acyl CoA. Two full-length cDNAs of *Arabidopsis* encoding proteins of 520 and 532 amino acids, respectively, were confirmed to encode acyl CoA:diacylglycerol acyl-transferases. Transformations of yeast and tobacco, respectively, with the *Arabidopsis* DGAT were performed. A 200–600-fold increase of DGAT activity in the transformed yeast was observed, which led to a 3–9-fold increase of TAGs accumulation. In the transformed tobacco, TAG content increased to 7-fold higher than that of a control plant. In addition, lipid droplets formation occurred in the cytoplasm of young growing leaf cells as a result of this transformation (Bouvier-Nave et al., 2000). DGAT gene has also been overexpressed in the plant *Arabidopsis* and it was shown that the oil content was enhanced in correlation with the DGAT activity, which increased by 10–70% (Jako et al., 2001).

The success with DGAT could be explained by the fact that the substrate of DGAT, diacylglycerol, could be allocated to either phospholipid biosynthesis or TAG formation. Overexpression of DGAT would commit more diacylglycerol to TAG formation rather than phospholipid formation. In fact, studies with plants have revealed that increasing the rate of TAG synthesis by overexpressing DGAT also stimulated the formation of fatty acid (Galili and Hofgen, 2002). All these results seem to suggest that the reaction catalyzed by DGAT is an important rate-limiting step in lipid biosynthesis. However, no reports regarding the overexpression of this enzyme in microalgae were located.

3.3. Overexpression of enzymes relevant to lipid biosynthesis

A few enzymes that are not directly involved in lipid metabolism have also been demonstrated to influence the rate of lipid accumulation by increasing the pool of essential metabolites for lipid biosynthesis. The following are a few examples.

3.3.1. The acetyl-CoA synthase (ACS)

ACS catalyzes the conversion of acetate into acetyl-CoA. It was observed that when growing a bacterial strain on acetate, overexpression of ACS could increase the rate of fatty acid synthesis. For instance, it was observed by Lin et al. (2006) that, by overexpressing the *acs* gene in *E. coli*, the ACS activity was increased by 9-fold, leading to a significant increase of the assimilation of acetate from the medium, which can contribute to lipid biosynthesis. This concept was also shown to be applicable to the secreted acetate during bacterial growth (Brown et al., 1977).

3.3.2. Overexpression of malic enzyme (ME)

The effect of ME, which catalyzes the conversion of malate into pyruvate and simultaneously reduces a $NADP^+$ molecule into NADPH, was studied in filamentous fungi in correlation with lipid accumulation (Wynn et al., 1999). It was observed that the enhanced energy (NADPH) supply as a result of ME overexpression was utilized by the enzymes involved in TAG synthesis and led to enhanced lipid production. It was observed that the enhanced activity of ME led to the increase of the cytosolic NADPH pool (i.e., the reducing equivalent that reflects the cellular energy state), making available more reducing power for lipogenic enzymes such as ACC, FAS and ATP:citrate lyase (ACL). It was proposed that a metabolon formation between FAS and ME could take place to create a channelling of the NADPH formed by ME toward the FAS active sites.

A similar strategy, i.e., to allow lipogenesis to occur without energy restriction by overexpressing ME so that the lipid accumulation becomes maximal, was investigated recently by Zhang et al. (2007) with *Mucor circinelloides*. The genes coding for ME from *M. circinelloides* (malEMt) and from *Mortierella alpine* (malEMc), respectively, were overexpressed in *M. circinelloides*. 2- and 3-fold increases in ME activity were observed for the transgenic malEMt and malEMc strains, respectively. In both cases, the ME activity increase was associated with a faster lipid accumulation. The amount of synthesized lipids was 2.5- and 2.4-fold higher for the transgenic malEMt and malEMc strains, respectively.

3.3.3. The ATP:citrate lyase (ACL)

ACL catalyzes the conversion of citrate into acetyl-CoA and oxaloacetate, and thus represents a source of acetyl-CoA for fatty acid biosynthesis. It has been well established that ACL is a key enzyme in lipid accumulation regulation in mammals, oleaginous yeast and fungi. It was also demonstrated that heterologous ACL can be imported into the plastids of plants. Rangasamy and Ratledge (2000) did an interesting experiment in which a gene encoding a fusion protein of the rat liver ACL with the leader peptide for the small subunit of ribulose bisphosphate carboxylase was constructed and introduced into the genome of tobacco. This was sufficient to transport the heterogonous enzyme into the plastids. In vitro assays of ACL in isolated plastids showed that the enzyme was active and synthesized acetyl-CoA. Overexpression of the rat ACL gene led to a 4-fold increase in the total ACL activity; this increased the amount of fatty acids by 16% but did not cause any major change in the fatty acid profile.

3.4. Blocking competing pathways

From the metabolic engineering point of view, blocking off competing pathways may also enhance the metabolic flux being channelled to TAG biosynthesis.

β-Oxidation is the principal metabolic pathway responsible for the degradation of fatty acids in eukaryotes (Shen and Burger, 2008). By doing so, it consumes fatty acids, the precursors of TAG formation. It is therefore possible to enhance TAG production by blocking this pathway. Cao et al. (2006) demonstrated that using an indirect method, i.e., inhibiting the acetyl-CoA transportation system required for coupling the β-oxidization in peroxisome and the TCA cycle in mitochondria but not any enzyme of the β-oxidation, is capable of inhibiting the β-oxidization of *Candida tropicali*. Dicarboxylic acids (DCAs) can be obtained by oxidizing alkanes by *C. tropicalis*. However, DCAs may be degraded to acetyl-CoA by β-oxidation, resulting in a limited DCA yield. In *C. tropicali*, acetyl-CoA can be transported into the mitochondrion for the TCA cycle by carnitine acetyl-transferase (CAT), by which the energy generation and β-oxidation are connected. It was shown that the reduction of the specific activity of CAT in recombinant cells by about 50% resulted in a 21.0% increase of the DCA concentration, and a 12% increase of the molar conversion of alkane. However, recombinants with no detectable CAT activity could not grow on alkane. These results indicate that partial inhibition of β-oxidation can facilitate DCA production. However, complete blocking of the transportation process would be harmful for energy supply. Picataggio et al. (1992) blocked β-oxidation in *C. tropicalis* by knocking out the genes encoding for acyl-CoA oxidase. It was observed that, not surprisingly, the growth of the cells was adversely affected.

Phospholipid biosynthesis is another competitive pathway to TAG formation because it competes against TGA biosynthesis for a common substrate, phosphatidate. If phosphatidate is converted into CDP-diacylglycerol instead of diacylglycerol, it enters the phospholipids synthetic pathway (Coleman and Lee, 2004). As mentioned previously, overexpression of the enzyme DGAT has the effect of channelling phosphatidate to TGA accumulation. On the other hand, it was shown that inhibition of phospholipid synthesis caused the formation of abnormally long fatty acids, due to supplementary elongation cycles (Jiang and Cronan, 1994).

The third competitive pathway is the conversion of phosphoenolpyruvate to oxaloacetate, which is catalyzed by the phosphoenolpyruvate carboxylase (PEPC). TAG biosynthesis also requires phosphoenolpyruvate (which converts successively to pyruvate, acetyl-CoA, malonyl-CoA and then fatty acids) (Song et al., 2008). By expressing antisense PEPC in *B. napus*, Chen et al. (1999) achieved a 6.4–18% increase in oil content, suggesting that reduced PEPC activity enhanced the lipid accumulation. Significantly enhanced lipid contents were also obtained with transgenic soybean lines harbouring anti-PEP gene (Sugimoto et al., 1989; Zhao et al., 2005). In microalgae, preliminary results also indicate that PEPC plays a role in the regulation of fatty acid accumulation and reduced PEPC activity by antisense expression was correlated with an increase of the lipid content in *Synechococcus* sp., a cyanobacterium (Song et al., 2008).

3.5. The multi-gene approach

The multi-gene approach, i.e., overexpressing more than one key enzymes in the TAG pathway to enhance lipid biosynthesis, was suggested by a few researchers (Roesler et al., 1997; Verwoert et al., 1995). However, literature on the feasibility of this strategy is scarce, probably due to the difficulties in manipulating multiple genes.

In summary, extensive studies have established a solid understanding of the lipid metabolism in different species. Based on the knowledge, numerous trials have been carried out to investigate the feasibility of manipulating the genes of key enzymes relevant to lipid synthesis to enhance lipid production of different species. They can be broadly classified into four different approaches: (1) overexpressing rate-limiting enzymes of the TAG biosynthesis pathway; (2) overexpressing enzymes that enhance the TAG pathway; (3) partially blocking competing pathways; and (4) the multi-gene transgenic approach. It seems that DGAT and ME are the most likely enzymes that might lead to enhanced lipid production when overexpressed in plants. However, no report was found regarding the overexpression of either of these two enzymes in microalgae. While completely blocking a competing pathway seems to be harmful to cell growth, preliminary successes have been achieved with the partial repression of CAT and PEPC. Of particular interest, reducing PEPC activity by expressing antisense gene was observed to be beneficial for lipid production in microalgae.

4. The transcription factor engineering approach

It was recently suggested that the regulation of metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level. The use of regulatory factors such as transcription factors (TFs) to control the abundance or activity of multiple enzymes relevant to the production of desired products has provoked widespread interests (Capell and Christou, 2004). This approach is referred to as TFE and can be more precisely described as a novel technology employing the overexpression of TFs that up- or down-regulate the pathway(s) being involved in the formation of target metabolites for the overproduction of them.

TFs are proteins that regulate DNA transcription by recognizing specific DNA sequences and establishing protein–DNA and protein–protein interactions. They have been classified into more than 50 families according to their conserved structure and their DNA binding domains. They can interact with the transcription machinery such as DNA polymerase and so to activate it to enhance the rate of transcription of a particular group of genes (Grotewold, 2008). They can also act as repressors or make subtle down-regulation changes in a metabolite production without repressing it totally. Quite frequently, a combination of TFs may regulate a single metabolic pathway (Santos and Stephanopoulos, 2008).

In contrast to the GE approach that targets a single gene, the TF approach affects a large number of genes involving multiple metabolic pathways, resulting in an integrated up- or down-regulation of these pathways simultaneously (Grotewold, 2008; Santos and Stephanopoulos, 2008). The emerging of "secondary bottlenecks", which is one of the major concerns of the GE approaches, is therefore less likely. This emerging metabolic engineering approach has been demonstrated to be able to improve the production of valuable metabolites (see Table 2) and represents an attractive alternative that is likely to bring out the breakthroughs in producing metabolically engineered microalgal strains for cost-effective TAG production.

Although TFE in microalgae is still in its embryo, numerous TFs have been shown to be able to stimulate the overproduction of valuable metabolites in different species and various TFs for the regulation of lipid synthesis in animals, plants and microorganisms have been identified. These results may provide valuable hints to TFE for enhanced microalgal lipid production.

4.1. Enhanced metabolite production by TFE

4.1.1. Zinc-finger protein transcription factors for enhanced pharmaceutical proteins

Zinc-finger protein transcription factors (ZFP TF), which typically contain many fingers linked in a tandem fashion, are some of the most extensively studied DNA-binding proteins. The zinc finger domain enables different proteins to interact with or bind DNA, RNA, or other proteins, and is present in the proteomes of a variety of different organisms. There are many types of zinc finger proteins, which are classified according to the number and order of their Cys and His residues that bind the Zinc ion. Among these, the C2H2-type zinc finger proteins, which have 176 members in *Arabidopsis*

Table 2
Transcription factors enhanced production of high-value products.

TF	Source-species (taxonomy)	Receiver-species (taxonomy)	Effectiveness	Refs
Artificial zinc fingers	Artificial	Tobacco (plant)	High level activation of a β-glucuronidase gene stable, inheritable, non-toxic	Segal et al. (2003)
Zinc fingers	Human	CHO cells	2-fold increase of IgG antibody production	Reik et al. (2007)
MYB and bHLH	*Arabidopsis thaliana* (plant)	*Arabidopsis thaliana* (plant)	Strongly enhanced flavonoid biosynthesis	Vom Endt et al. (2002)
ORCA2	*Catharanthus roseus* (plant)	*Catharanthus roseus* (plant)	Induction of genes leading to TIA biosynthesis	Vom Endt et al. (2002)

thaliana alone, constitute one of the largest families of TFs in plants. They are mostly species-specific and contain a conserved QALGGH sequence within their zinc finger domain. Recent functional characterization studies of different C2H2 proteins in *Arabidopsis* suggest that many of these proteins function as part of a large regulatory network that senses and responds to different environmental stimuli (Ciftci-Yilmaz and Mittler, 2008). Based on the understanding to the structural and functional features of naturally occurring zinc finger proteins, several design strategies have been proposed for the creation of artificial zinc-finger proteins for applications in gene regulation and gene therapy (Negi et al., 2008; Segal et al., 2003; Stege et al., 2002). Enhanced production of a therapeutic protein was achieved by overexpressing a ZFP TF that binds a DNA sequence within the promoter of a therapeutic protein from mammalian production cell lines (Reik et al., 2007). This ZFP TF enabled more than 100% increase in protein yield from CHO cells. Expression vectors engineered to carry up to 10 ZFP binding sites further enhanced ZFP-mediated increases in protein production up to 500%.

4.1.2. MYB and bHLH transcription factors for enhanced production of flavonoids in plants

MYB and bHLH transcription factors have been studied in plants such as *A. thaliana* and have been demonstrated to regulate the biosynthesis of flavonoids, more precisely anthocyanin and seed coat tannin (Vom Endt et al., 2002). When genes R and C1, which encoded a bHLH and a MYB protein, respectively, were ectopically expressed in normally unpigmented cell lines, accumulation of anthocyanin was observed. This was the consequence of a coordinate response to the TFs in the form of a global expression of the structural genes (Vom Endt et al., 2002). Similarly, overexpression of MYB in *Arabidopsis* caused a significant enhancement of the flavonoid biosynthesis (Vom Endt et al., 2002).

4.1.3. ORCA2 protein for enhanced alkaloid production in plants

Plant alkaloids are a source of many novel natural products such as pharmaceuticals. Several TFs involved in the regulation of plant alkaloid biosynthesis genes have been isolated and studied. Among them is ORCA2 protein, the TF stimulates terpenoid indole alkaloid (TIA) biosynthesis. It was shown that by overexpressing ORCA2, multiple genes of the TIA biosynthesis pathway were overexpressed, leading to an increase of TIA formation (Vom Endt et al., 2002).

4.2. TFE for enhanced microalgal lipid production: how far are we from there?

To be able to implement the TFE strategy for lipid overproduction in microalgae, TFs from algae have to be identified. A few TFs have been identified as responsible for the regulation of lipid biosynthesis in animals and plants. For instance, sterol regulatory element binding protein (SREBP) has been established as the master regulators of lipid homeostasis in mammals (Eberle et al., 2004; Espenshade, 2006; Espenshade and Hughes, 2007; Goldstein et al., 2006; Hitoshi, 2005; Horton, 2002; McPherson and Gauthier, 2004; Porstmann et al., 2005; Todd et al., 2006; Yang et al., 2000). In plants, it was demonstrated that SebHLH protein, a member of the bHLH family TFs, might play a key role in the transcriptional regulation of genes related to storage lipid biosynthesis and accumulation during seed development (Kamisaka et al., 2007). It was also reported that soybean Dof-type (DNA binding with one finger) TF genes were involved in the regulation of the lipid content in soybean seeds. Among the 28 Dof-type TF genes in soybean plants, which displayed diverse patterns of expression in various organs and exhibited different abilities for transcriptional activation and DNA binding, two genes, GmDof4 and GmDof11, were found to increase the content of total fatty acids and lipids in transgenic *Arabidopsis* seeds by upregulating genes that are associated with the biosynthesis of fatty acids (Wang et al., 2007a). The Dof-type TF family sequences were also identified from a variety of representative organisms from different taxonomic groups: the unicellular green alga *Chlamydomonas reinhardtii*, the moss *Physcomitrella patens*, the club moss *Selaginella moellendorffii*, the gymnosperm *Pinus taeda*, the dicotyledoneous *A. thaliana* and the monocotyledoneous angiosperms *Oryza sativa* and *Hordeum vulgare* (Moreno-Risueno et al., 2007). It is worth noting that SREBP proteins, the master regulator of mammalian lipid homeostasis, are also found in plants and microorganisms with high conservation of sequences (Bengoechea-Alonso and Ericsson, 2007; Espenshade and Hughes, 2007; Todd et al., 2006). Nevertheless, their regulatory functions are very different from those in mammals. For instance, in fission yeast *Schizosaccharomyces pombe*, the SREBP analog, which is called Sre1p, was found to be a principal activator of anaerobic gene expression, upregulating genes required for nonrespiratory oxygen consumption, among many other upregulated genes, while down-regulating a large number of other genes. It was observed that oxygen-requiring biosynthetic pathways for ergosterol, heme, sphingolipid, and ubiquinone were the primary targets of Sre1p, which acted directly at target gene promoters (Todd et al., 2006).

TFs can be classified into a pyramidal hierarchy, where the high-level TFs influence the low-level ones (Grotewold, 2008). The predictability of the TFs is variable and depends on their level in the hierarchy. A high-level TF has important impacts on other TFs and in consequence regulates a broad range of genes. It follows that, quite logically, predicting the outcome of these high-level TFs on global gene expression is still troublesome with our limited understanding at present. On the other hand, low-level TFs are generally less conserved and are hence much more difficult to be employed in inter-species metabolic engineering manipulations. It seems to be clear from the previous discussion that the TFs regulating lipid biosynthesis are low-level TFs as different species have different TFs for lipid regulation. There is so far not much information regarding the lipid regulation TFs of microalgae.

At least 147 putative TFs and 87 putative transcription regulators (TRs) (proteins that assist TF functions) have been identified in the green alga *C. reinhardtii* up to 2008. However, only the biological functions of a small number of them have been determined (Riano-

Pachon et al., 2008). As aforementioned, no literature regarding the TFs regulating lipid biosynthesis in microalgae was found. The most important task at present is therefore to identify lipid-regulating TFs of microalgae should we plan to exploit the TFE strategy for enhanced lipid production. Fortunately, various technologies for the identification, purification, and characterization of TFs have been developed, providing a solid foundation for future studies in this direction.

A common strategy used for the identification of TFs involves comparing the transcriptomics and proteomics of target microalga under controlled conditions that allow and prohibits the formation of metabolites of interest, respectively. For instance, Egan et al. (2002) identified a ToxR-like transcription regulator (WmpR) that controls the expression of fouling inhibitors in *Pseudoalteromonas tunicate* by analysing the gene sequence of a transposon mutant deficient in antifouling activities and comparing the proteomics of the wildtype and the mutant strains using 2D-PAGE. Then, more precise analyses unveiling the regulator functions of WmpR were carried by Stelzer et al. (2006) using the combination of proteomic analysis (2D-PAGE) and transcriptomic studies (RNA arbitrarily primed PCR). Recently, transcriptomic studies using microarrays were employed by Nguyen et al. (2008) to identify factors regulating the hydrogen production of *C. reinhardtii*. In this study, microarray analyses were used to obtain global expression profiles of mRNA abundance in the green alga *C. reinhardtii* at different time points before the onset and during the course of sulfur-depleted hydrogen production. These studies were followed by real-time quantitative reverse transcription-PCR and protein analyses. Among the more than 2-fold differentially expressed genes, 10 were classified as having a putative role in transcription or translation. Four of these were up-regulated during the hydrogen production phase, including the genes encoding pre-mRNA processing factor 3 (PRP3), the eukaryotic initiation factor 4A-10 (eIF4A-10), splicing factor 3a subunit 3 (SP3a3), and the chloroplast 30S ribosomal protein L11 (rps11).

A number of different techniques have been developed for purification of TFs, which is an important step prior to studies on the structure and functions of TFs (Jiang et al., 2007; Maouche and Cohen-Kaminsky, 1997; Schulman and Setzer, 2002; Yang, 1998). In principle, these methods are all based on the ability of TFs to recognize and interact with specific DNA sequences present in the promoters of eukaryotic genes. The purification of a TF begins with the preparation of nuclear extracts from appropriate cells or tissues (Gorski et al., 1986), which is then subjected to a series of pre-treatment procedures (Yang, 1998) if necessary, followed by DNA affinity chromatography (Moxley and Jarrett, 2005).

Various technologies have been employed for characterizing the structure of TFs and their interaction with DNA sequences. These technologies include electrophoretic mobility shift assay (EMSA) (Hattori et al., 2007; Hickman and Harwood, 2008; Wang et al., 2003; Yang, 1998), the DNase I protection (footprinting) assay (Wang et al., 2007b; Yang, 1998) and the Southwestern blotting (Wang et al., 2003; Yang, 1998). NMR spectroscopy (Bagby et al., 1998; Yamasaki et al., 2008) and X-ray crystallography (Burley and Kamada, 2002; Yamasaki et al., 2008) are also commonly used in combination with other methods to study TF structure and TF–DNA interaction. A comprehensive knowledge of TF binding sites (TFBS) is important for the understanding of TF regulatory functions. Techniques for identifying TFBS, including experimental techniques and computational approaches, can be found in a few recent reviews (Efromovich et al., 2008; Elnitski et al., 2006; Hannenhalli, 2008; Marinescu et al., 2005; Merkulova et al., 2007; Mukhopadhyay et al., 2008).

In summary, TFE method is a very promising technology that is likely to bring about the necessary breakthrough enabling cost-effective microalgal oil production. However, TFE for enhanced microalgal lipid production is still in its embryonic stage and the important first step would be to identify TFs regulating microalgal lipid biosynthesis. Various technologies are available for the identification, purification, and characterization of TFs and TF functions.

5. Conclusion

There are three promising strategies that could potentially be employed for enhancing lipid production of microalgae, the BE strategy, the GE strategy, and the TFE strategy. Firstly, the BE strategy, which relies on applying physiological stresses such as nutrient-depletion to channel metabolic flux to lipid biosynthesis, is the most mature and most widely employed among the three at present. Secondly, the GE and the TFE strategies are more promising in a long-term perspective. Although there is a lack of success stories in lipid overproduction using transformed microalgal strains, the knowledge obtained in studies on lipid pathways and genetic transformed organisms for enhanced lipid synthesis among other species suggests that DGAT and ME are the most promising targets for gene transformation. Down-regulation of PEPC gene to reduce the PEPC activity was also suggested to be beneficial for lipid production in some microalgal species. Finally, TFE is an emerging technology that has a great potential. In-depth studies on the physiological functions of microalgal TFs and identification of TFs regulating lipid pathways of different microalgal species are the essential steps for successful implementation of the TFE strategy. To this end, a number of techniques have been developed for the identification, purification, and characterization of TFs.

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