Genetic manipulation of microalgae for improvement of biodiesel production

Supachai Vuttipongchaikij

Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand

E-mail: fsciscv@ku.ac.th

ABSTRACT

Microalgae are considered the most promising source for biodiesel production for the near future. Various characteristics that promote their potential include high and rapid biomass production rate, ability to accumulate high amount of lipid up to 80% of their cell mass, low cultivation cost and no competition for arable land for crop plants. Selection of high performance strains is the important key for efficient biodiesel production with competitive production cost. However, strains isolated from nature so far only possess one or a few of the required characteristics. Therefore, they are insufficient for the performance demands for biodiesel production. To obtain microalgal strains that are comprised of the required key characteristics, genetic manipulation is the most plausible approach to reach this goal. In this review, key characteristics of microalgae that are required for efficient biodiesel production are discussed for their possibility to be manipulated by either mutagenesis or gene transformation. Forward and reverse genetics in microalgal system in functional genomic era are described regarding the benefits obtained from genome sequences that will contribute to gene function analysis and algal strain improvement. Furthermore, each genetic transformation method is described regarding its pros and cons. Finally, the bottlenecks in many aspects of microalgal strain improvement are underlined as future challenges.

Keywords: microalgae, biodiesel, genetic transformation, forward genetics, reverse genetics

INTRODUCTION

With the current world energy crisis due to dwindling crude oil supply and high fuel costs, alternative sources of energy have been surveyed with a focus on economic feasibility and environmental friendliness. Among others, microalgae are considered the most promising source of clean and renewable energy (Chisti, 2007, 2008). Although the cost effectiveness is not yet competing with that of petroleum fuels, the production of biodiesel from microalgae is currently operated in many countries, demonstrating its potential as our future energy source (Wijffels and Barbosa, 2010). Microalgae have been promoted mainly because of four following key advantages; (i) A

number of microalgal species have high biomass production rate that exceeds that of land plants (Mirón *et al*., 1999); (ii) Upon starvation, microalgae accumulate various compounds for their survival. These are mainly in the forms of triacylglycerides (TAG) and starch, which are perfect substrates for biodiesel and bioethanol productions, respectively. Many species of microalgae isolated from nature accumulate TAG as oil droplets within the cell and some species contain exceptionally high amount of lipid, as much as 80 % in dry mass (Banerjee *et al*., 2002; Chisti, 2007; Rodolfi *et al*., 2009). Furthermore, there is a line of evidence suggesting that crude oil might be produced by certain species of microalgae, possibly diatoms, and, thus, biofuel production from these microalgae could be compatible with our current petroleum fuel production process (Ramachandra *et al*., 2009); (iii) Growing microalgae requires minimal amount of nitrogen and phosphate supplies and CO_2, and they could be easily cultivated in various types of water including industrial-treated wastewater and seawater (Benemann and Oswald, 1996). This could greatly reduce the cost of production and makes it economically feasible; (iv) Cultivation of microalgae does not compete with agricultural production in terms of arable land requirement and food price unlike other biofuel sources such as palm oil, rapeseed oil, corn, sugar cane and cassava (Cassman and Liska, 2007).

The choice of algal strains is one of the key factors that can greatly affect the production cost. Although fast-growing and high-lipid content strains can be isolated from nature, several barriers still need to be overcome before algal biodiesel production can be economically viable. Furthermore, an ideal algal strain needs to be able to tolerate variable environmental conditions including high light intensity from midday sun, which normally oversaturates the photosynthetic cycle, limits growth and reduces productivity (Rubio *et al*., 2003). Additionally, other abiotic stress factors such as temperature, pH and salt concentration, combined with high oxygen level generated from photosynthesis, lead to the production of reactive oxygen species (ROS), which cause cell damage and ultimately lead to cell death. Thus, the ideal strain needs to have high tolerance to abiotic stress and oxidative stress (Ledford and Niyogi, 2005). Fluctuations of conditions during growth period also influence the biomass yield especially in an open pond system, which is highly variable in light intensity and temperature. Therefore, strains that are best adaptive to environmental changes would be preferable because they can continuously grow without dropping their growth rate (Grobbelaar, 2000). Having large cell size with fast doubling time is also desirable as, presumably, the amount of accumulated lipid will increase with more biomass (Borowitzka, 1992). The strain itself needs to be able to efficiently synthesize and accumulate lipid. If the strain can secrete the lipid outside the cell, the need for expensive and complicated process for harvesting and extraction will no

longer be required. If lipid needs to be extracted from the cell, a useful trait for the ideal strain is the ability to form flocs (Borowitzka, 1997). Freely suspended algal cells float around in the growth medium and separation requires expensive centrifugation with high-energy demand. Aggregates of algae or flocs can be recovered by sedimentation rather than centrifugation, thus, reducing cost and energy consumption for harvesting.

After decades of searching for suitable microalgal strains throughout the globe, it has come to a conclusion that such ideal strains are unlikely to be found in nature. Most strains known to date possess only one or a few characteristics, each within a limited range. Unlike crop plants that have been improved by breeding programs to meet certain required traits, the availability of microalgae is limited to strains that have been isolated. Therefore, genetic manipulation is a solution for obtaining ideal strains with different desired characteristics as suggested by several authors (Mata *et al*., 2010; Radakovits *et al*., 2010; Wijffels and Barbosa, 2010). In this review, several approaches to improve productivity, lipid yield and other key traits that would be beneficial to biodisel production are described. The genetic approaches both forward and reverse are considered in the context of functional genomic era. Finally, practical methods for genetic manipulation are discussed for future applications in microalgal system.

Key microalgal traits to be improved for high efficiency biodiesel production and the possibility of using genetic manipulation

Microalgal traits that would dramatically facilitate and enhance the process of biodiesel production can be summarized into (i) fast growth and, perhaps, large cell size for high biomass production, (ii) high lipid yield, (iii) the ability to secrete lipid into media, (iv) adaptive capability to environmental fluctuations and stress and (v) the ability to form flocs for easy and low cost harvesting (Zittelli *et al*., 2006). In order to achieve these traits by genetic manipulation, understanding the basis of the genetic control for each trait is needed. With our current understanding of these biological processes, we can anticipate the possibility of using either genetic engineering or mutagenesis for improvement each of the key microalgal traits.

Fast growing microalgae with large cell size is attractive for biodiesel production because increased amount of biomass means an increase in oil production. However, genes responsible for these traits are mostly unknown. Additionally, these traits are quantitative characteristics and are likely under the control of multiple genes. Overexpression of only one or two known genes is unlikely to achieve the goals. Screening mutagenized populations is a more preferable alternative, which could provide strains with large cell size and rapid cell cycle. Large cell size could be obtained

through endo-mitosis or endo-reduplication, which is a process that increases ploidy levels without further cell division. This has been previously done in various organisms including algae; however, the process is limited to certain species (Nagl, 1978; Sugimoto-Shisaru and Roberts, 2003). However, a huge task in screening process is the main barrier. Methods for identifying strains with ability to grow faster or having large cell by analyzing individual cell among the mutant population are extremely laborious. The solution for this task might lie on the future progress in identifying genes involved in controlling the rate of cell division and cell size.

For direct gene transformation, increasing lipid yield has been the prime target for microalgae improvement and research. However, many attempts have been reported without success. A number of genes involved in fatty acid biosynthesis pathways have been exploited in these studies. Heterologous overexpression of Acetyl CoA carboxylase (ACCase), a key enzyme in controlling fatty acid biosynthesis, from various organisms including *Brassica napas* (Topfer *et al*., 1995) and diatoms failed to significantly increase total oil content in the cells, despite the two-to-three-fold increase in the ACCase activity (Dunahay *et al*., 1996). Overexpression of spinach *3-ketoacyl-acyl-carrier protein synthase* (*KAS III)*, encoding an enzyme catalyzing the rate limiting step in fatty acid biosynthesis, even resulted in a slight decrease of lipid yield (Dehesh *et al*., 2001). Furthermore, overexpression of the endogenous orthologs of diacylglycerol acyltransferase encoding enzymes in TAG biosynthesis, *CrDGAT2a*, *CrDGAT2b*, and *CrDGAT2c*, in *Chlamydomonas* also failed to increase the total oil content (La Russa *et al*., 2012), even though overexpression of *DGAT1* in plant systems has been shown to significantly increase seed oil contents (Jako *et al*., 2001). These examples suggest thatthe process of fatty acid and lipid biosynthesis pathway in microalgae might be complex and that the building blocks in each step are subjected to rate-limiting steps with unknown feedback regulations. Therefore, overexpressing a single gene in lipid biosynthesis is unlikely to increase the lipid yield. Nevertheless, mutagenesis study in *Chlamydomonas* had shed some light on increasing lipid production. Analysis of *Chlamydomonas* strains with the starchless phenotype showed that the mutant that is defective in ADP-glucose pyrophospho-rylase, an enzyme in starch biosynthesis pathway, displayed a two fold increase in the total amount of lipid droplet compared to wildtype (Wang *et al*., 2009). This increase is due to the diversion of starch biosynthesis to TAG biosynthesis caused by the mutation. This finding demonstrates the possibility in obtaining higher lipid producing strains that are resulted from alternations in molecular processes by random mutagenesis.

The possibility to genetically engineer microalgae to excrete lipid for ease of harvesting is supported by the identification of genes involved in the secretory pathway in

other organisms. A disruption in acyl-CoA synthetase in *S. cerevisiae* mutant obtained from a forward genetic screen was shown to cause lipid secretion in yeast (Michinaka *et al*., 2003). Such excretion is likely due to enzyme disruption causing the accumulation of fatty acid within the cell to reach toxic level, which in turn triggers the export mechanisms. A more likely mechanism for generating microalgal strains that excrete lipid may lie with ATP-binding cassette (ABC) transporters. In plants, these proteins are involved in exporting various compounds including heavy metals, auxin and ions (Pighin *et al*., 2004; Schulz and Kolukisaoglu, 2006). In *Chlamydomonas* genome, there are more than 100 open reading frames containing ABC domain and not much is known about ABC transporters in microalgae (Hanikenne *et al*., 2005). This is an open opportunity for characterizing and exploiting these genes for engineering lipid secreting strains. However, this does not preclude the opportunity in obtaining lipid-secreting strains from random mutagenesis and screening scheme.

Stress tolerance and adaptation would benefit cell growth in different growth conditions especially in outdoor ponds where many growth parameters are fluctuated over time. Versatile strains would grow better and, hence, provide faster growing culture. Previous screening of mutagenized microalgae has yielded many mutants with various tolerant characteristics. The robustness of this approach is due to its straightforward screening strategies using stress conditions that select only the tolerant strains. One example that shows an increase in tolerance to high light intensity is the *Chlamydomonas* mutant with reduced antenna size and higher photosynthetic efficiency (Polle *et al*., 2000; Tetali *et al*., 2007). Several isolated mutants have been shown to accumulate high levels of antioxidants and are tolerant to conditions that induce oxidative stress (Baroli *et al*., 2003). Manipulation of genes that are responsible for stress tolerance has been shown to enhance the ability of microalgae to endure various conditions as well. Overexpression of *chloroplast small heat shock protein* (*ch-sHSP)* in *Synechococcus elongates* resulted in higher thermotolerance under light condition than the wild-type strain (Nakamoto *et al*., 2000). *HSP70B*-overexpressing *Chlamydomonas* exhibited greater photosynthetic efficiency because of the protection of photosystem II (Schroda *et al*., 1999). Furthermore, expression of genes that enhance antioxidant production also promotes stress tolerance. The overexpressors that accumulate about two times the usual amount of xanthophyll were more resistant to oxidative stress conditions induced by high light intensity and high temperature (Davison *et al*., 2002). Overexpression of *homogentisate phytyltransferase vitamin E2* (*VTE2)*, the first-committed step of tocopherol synthesis, led to higher protection against oxidative stress (Li *et al*., 2012). Moreover, enzymes that recycle antioxidants such as dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) have also been

overexpressed, though in plant systems, and resulted in higher tolerance to various abiotic stresses such as salt, cold, drought and ozone (Le Martret *et al*., 2011). This suggests that it is highly possible to use genetic engineering to enhance the stress tolerance in microalgae through the use of genes either involved with antioxidants or stress response mechanisms.

Creating flocculating microalgae is challenging because flocculation is caused by interactions between cell wall and membrane proteins of each microalgal cell in the culture. Although there are many microalgal species that possess this ability, mimicking flocculation of an organism in another by expressing a few genes is unlikely to yield the expected result. Additionally, molecular mechanisms controlling flocculation in microalgae are barely known. Screening for flocculating strains from mutagenized population is somewhat unlikely to be successful because introducing a specific binding interaction between the cells could be limited by the genetic basis of the organism used. The best understood flocculation process is in yeast (Verstrepen *et. al*., 2003). This process employs the binding of a glycosyl binding protein called flocculin, which is immobilized on the yeast cell wall, to mannose moieties of cell wall glycoproteins. However, the understanding of flocculation in yeast system cannot be directly applied to microalgae since the basis of proteins and other molecules in the cell walls in both organisms are quite different. Further characterization of specific genes involved in flocculation in microalgae is needed to create a flocculating algal strain.

Forward and reverse genetics in functional genomic era: systematic approaches for microalgae improvement

Forward genetic screening of microalgal mutants arising from random mutagenesis studies including UV irradiation or chemical-induced mutations has been a powerful tool for obtaining new strains with desirable phenotypes (Fig. 1a). Various phenotypes that benefit biodiesel production can be selected upon depending on the selection criteria. Subsequent analysis of the genetic basis of the mutations, especially in *Chlamydomonas*, has led to the discovery of numerous gene functions. The benefit of this approach is that new algal strains could be obtained without previous understanding in the genetic basis of the algal species. The target strains are screened and tested by different screening criteria, and the selected strains can be directly used for their applications. However, identifying the mutated gene may be challenging. Until very recently, the identification of point mutations has to be carried out through map-based cloning, which is very laborious and requires crossing and genetic linkage verification (Rymarquis *et al*., 2005). *Chlamydo monas* is currently the only microalgal system that is routinely employed for gene functional analysis. This is because *Chlamydomonas* linkage map and the protocol for genetic

crossing have been extensively established (Kathir *et al*., 2003). Additionally, the complete *Chlamydomonas* genome sequence has boosted the gene identification process (Dent *et al*., 2005). However, gene identification of mutants from species other than *Chlamydomonas* by map-based cloning is almost impossible. Although the genome sequences of some microalgal species such as *Chlorella* are completed (Blanc *et al*., 2010), genetic crossing is not available in these species, and, therefore, linkage analysis cannot be performed.

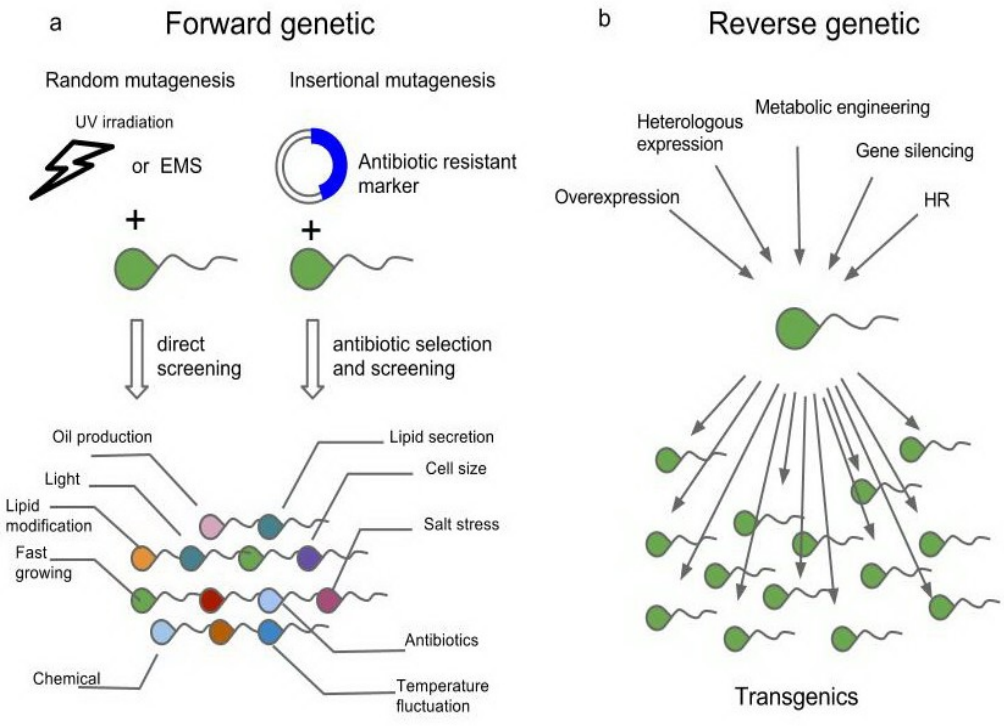

Figure 1 Schematics of forward and reverse genetic approaches for microalgal strain improvement. (a) A representation of forward genetics by using either random or insertional mutagenesis to generate a microalgal mutant population, which will be screened for desired phenotypes. (b) Using available characterized or annotated genes, improved microalgal strains can be generated through reverse genetics by gene expression, gene silencing or gene modification by homologous recombination (HR). See color figure on the website.

Insertional mutagenesis for gene inactivation is an alternative approach to obtain a mutant population for gene identification in various microalgal species (Tam and Lefebvre, 1993; Dent *et al*., 2005). Insertion of a DNA fragment into the coding region of a particular gene results in the loss of function of that gene. This process may yield strains with special

characteristics that could be selected through screening strategies. The advantages of using DNA fragment for insertion inactivation are the ease of mutant isolation and identification of insertion points of the DNA fragment within a genome (Walbot, 1992). This is because an antibiotic resistance gene is included within the inserting DNA fragment, and mutant strains could be selected based on their antibiotic resistance. Furthermore, the sequence on the DNA fragment can be used as a template for PCR analysis, sequencing or as a target for tagging probes for identifying the insertion point. A number of strategies have been developed for rapid identification of DNA insertion region (Gonzalez-Ballester *et al*., 2005).

The progress in genome sequencing expands the opportunity in identifying the genes from different algal species. Known genes from other organisms can be transferred to the desired microalgae through genetic manipulations to acquire traits that are important for biofuel production. Organelle genomes, especially the chloroplast genomes, were first available from *Chlamydomonas*. Shortly after that, numerous chloroplast genomes from many species of microalgae were made available (see Grossmann, 2005 for review). The sequences of nuclear genomes have been made publicly available at later stage. These included the red alga *Cyanidioschyzon merolae* (Matsuzaki *et al*., 2004), the diatom *Thalassiosira pseudonana* (Armburst*et al*., 2004), the green alga *Chlamydomonas reinhardtii* (Merchant *et al.,* 2007), and the picoeukaryote *Ostreococcus tauri* (Derelle *et al*., 2006). Furthermore, a number of transcriptomes and EST databases from different species such as *Chlamydomonas* and *Dunaliella* are available to facilitate the study of the algal genomes (Shrager *et al*., 2003; Rismani-Yazdi *et al*., 2011). With new and faster sequencing technology, genome sequences of many microalgae species are lining up to be released. Availability of gene and putative gene sequences will directly lead to the study of gene function by reverse genetic approach. In particular, this will benefit the improvement of traits that are controlled by multiple genes such as lipid production or stress responses.

Genetic improvement of microalgae is gradually moving into reverse genetic in which known gene sequences that are available in various databases can be used directly for trait improvements or for gene function studies (Fig. b). Ectopic expression or overexpression of endogenous genes is the simplest choice for this approach. Genes that either have been previously characterized or annotated without their precise functional roles can be expressed under the control of different promoters to obtain a desired level of gene expression. Alternatively, heterologous expression of genes from different organisms can be used to improve traits in selected microalgae. Moreover, with the availability of annotated genes in the databases, expression of a set of genes involved in a specific pathway could be a

potential approach for metabolic engineering (Beer *et al*., 2009; Lü *et al*., 2011). Apart from adding genes, the expression of gene within a genome can be altered for gene knockdown-knockout or gene switching by several methods. Gene silencing by RNAi has been used successfully for reducing gene expression level in microalgal systems (Rohr *et al*., 2004; Zhao *et al*., 2009). Although homologous recombination has been routinely used for genetic transformation in the chloroplast genome of microalgae, it has been previously considered to be impossible to perform on the nuclear genome of microalgae. However, a recent report for high-efficiency homologous recombination in *Nannochloropsis* has demonstrated the possibility of gene knockout and gene switching in specific microalgal species (Kilian *et al*., 2011). Thus, screening for microalgal species with the ability for homologous recombination could expand future genetic manipulation and application.

Methods for genetic transformation in microalgae

Various methods for gene transformation in microalgae that have been developed were primarily based on *Chlamydomonas*. They are either enzymatic or mechanical processes, which alters the cell wall structure of microalgae and enable the passage of DNA through the cell membrane. Other biological processes within the cells then mediate the integration of foreign DNA into the genome (Leon-Banares *et al*., 2004; Potvin and Zhang, 2010). The glass bead method has been extensively used for *Chlamymonas* transformation because of its simplicity and high efficiency. By simply vortexing the cells and DNA in the presence of glass beads and polyethylene glycol (PEG), a large number of transformants can be obtained (Kindle, 1990). However, prior to the transformation, cell walls have to be removed by autolysin treatment or a cell wall-less mutant has to be used (Kindle, 1990). Thus, this method is currently limited to *Chlamydomonas* because cell wall structures of other microalgae species are mostly unknown and the optimization of the transformation protocol for efficient cell wall removal is needed. Silicon carbide (SiC) whisker provides an alternative tool for glass bead. SiC could be mixed and vortexed with the cells without PEG and cell wall removal (Dunahay, 1993). Thus, it could be used for transformation of other microalgal species. Nevertheless, the short supply and health related concerns of SiC make it less preferable in research.

Electroporation has been adopted for *Chlamydomonas* transformation following the success of this system in various organisms including bacteria, yeast, fungi and mammalian cells (Chassy *et al*., 1988; Gietz and Woods, 2001). The application of the electric field increases either the permeability or the poration of the plasma membrane, enabling the DNA fragment to enter the cells. This method has been proven for its high efficiency compared to the glass bead method and can be used directly with intact cells (Shimogawara *et al*,

1998). Electroporation has been applied for transformation in many different microalgal species, although many parameters including electrical voltage, temperature, salt concentration or DNA concentration need to be verified for the successful transformation (Brown *et al*., 1991; Wang *et al*., 2007). In line with physical means of transformation, particle bombardment or gene gun, which uses high pressure to deliver the tungsten or gold particles coated with DNA into the cells, also has been used in microalgae such as *Haematococcus* (Teng *et al*., 2002) and *Chlorella* (El-Sheekh, 1999). Additionally, particle bombardment has been efficiently used for chloroplast transformation (Kindle *et al*., 1991). However, the high costs of bombardment equipments and for each operation limit its use (Boynton and Gillham, 1993).

Agrobacterium has been widely used for gene transformation in plant systems, and, has been shown recently to be applicable in other organisms including filamentous fungi (de Groot *et al*., 1998), mammalian cells (Kunik *et al*., 2001), and, particularly, several species of microalgae such as *Chlamydomonas, Haematococcus* and *Dunaliella salina* (Kumar *et al*., 2004; Kathiresan *et al*., 2009; Anila *et al*., 2011). The advantage of *Agrobacterium* transformation is that the DNA fragment is transferred and integrated directly into the microalgal genome through a biological process of *Agrobacterium* and the host cell, thus, providing stability of the transgene (Kumar *et al*., 2004). This provides a potential for scaling up the transgenic culture without using antibiotics. Furthermore, the copy number of the transgene seems to limit to only a few copies unlike high copy number integration introduced by electroporation or particle bombardment (Potvin and Zhang, 2010). This may be a benefit as the high copy number of transgene could sometimes leads to gene silencing and the low copy number provides stable gene expression. Moreover, the simple and low cost procedure that only requires co-cultivation of *Agrobacterium* and microalgae makes it an attractive method to be used for microalgal transformation.

Regulatory elements used in the expression cassettes or vectors for gene transformation in microalgae have also been developed based on *Chlamydomonas* transformation. Constitutive promoters that are the most commonly used in microalgae were developed from five prime untranslated regions of *Photosystem I complex protein* (*PsaD)* and *ribulose bisphosphate carboxylase 2 (RBCS2*) (Lumbreras *et al*., 1998; Fischer *et al*., 2001). Constitutive promoters used in plant systems such as 35S Cauliflower mosaic virus (35S CaMV) have also been shown to drive strong levels of expression in microalgae (Kumar *et al*., 2004). There are a number of promoters developed for inducible expression system such as promoter of the*cytochrome c6 (CYC6)* gene from *Chlamydomonas* and *nitrate reductase* genes from diatom *Cylindrotheca fusiformis*. The *CYC6* promoter is induced in the absence of copper ion in the media or by adding nickel

or a chelator such as EDTA into the media (Ferrante *et al*., 2008). The *nitrate reductase* promoters are induced by the addition of nitrate to the media (Poulsen *et al*., 2005). However, in the context of large-scale culture for biodiesel production, these promoters are not yet suitable as controlling the concentration of the inducers in large-scale production and adding specific compounds such as nitrate or chelating agents into the culture pond might not be economically feasible. Therefore, new promoters are needed for a wider application in microalgae transformation, especially those for lipid production and accumulation. Because oil production in microalgae usually occurs during the stationary phase that relates to the shortage of food supply or stress, promoters that drive gene expression in coordinate responses to these conditions would be useful for genetic engineering of lipid biosynthesis and oil production. A number of reports have shown a list of genes that are activated during nitrogen deprivation (Miller *et al*., 2010) or in response to NaCl (Sun *et al*., 2010). These could be the prime targets to be used as inducible promoters for oil production in microalgae.

Early versions of selectable markers were developed based on the homologous complementation strategy that enables the rescue of various auxotrophic *Chlamydomonas* mutants, which contain mutations in the corresponding marker genes. These include *Arg7*, *Nit1*, *Oee1* or *AtpC* (Kindle *et al*., 1998). This strategy strictly requires a specific mutant for a specific marker and is not applicable to the wild-type strain or diploid microalgae unless both alleles are defective. Selection of transformants using antibiotic resistant marker genes has been shown to be effective in microalgae, especially in *Chlamydomonas*. These include a group of *AphVIII* genes, which provides resistant capacity to paromomycin, kanamycin or neomycin (Sizova *et al*., 2001), *Ble* gene for bleomycin resistance (Stevens *et al*., 1996), and *Hpt* gene for to hygromycinB resistance (Hall *et al*., 1993). After a transformants obtained, the next critical issue to be addressed is whether the transgene is expressed transcriptionally and translationally. Reporter genes that can be used to indicate expression at the translational level have been developed for microalgae, including *uidA* (*GUS)* (Lohuis and Miller, 1998) and green fluorescence protein (*GFP)* (Furhmann *et al*., 1999). Alternatively, short polypeptide tagging could be used for indicating expression of the transgene. Short DNA coding sequences for short polypeptides such as HA and c-myc tags could be fused with the target gene, and the protein products can be detected by antibodies that recognize these short polypeptide tags.

Bottlenecks in genetic engineering of microalgae and future challenges

Although there are many transformation methods developed for microalgae, they have been optimized based on a few certain species, mostly *Chlamydomonas*, and cannot be directly used in other microalgal species without further evaluation and optimization. Applicability of

each method is different between microalgal species, and the suitable method for each species should be verified. For example, the glass bead method is highly efficient in *Chlamydomonas* transformation but yields a very poor transformation rate in other species (Coll, 2006). Therefore, when obtaining new species, an optimized transformation protocol has to be developed. Selection conditions, particularly the concentration of antibiotics, are needed to be carefully examined to ensure efficient transformant selection.

The instability of the transgene is commonly observed after several rounds of cultivation in non-selective media. This could be because there is no integration of the transgene into the algal chromosome and the transgene is episomally replicated and maintained in the algal cells through selection condition (Rochaix and van Dillewijn, 1982; Boynton *et al*., 1988). A number of authors suggested that this problem could be solved by screening more transformants (Kindle, 1998; Schiedlmeier *et al*., 1994).

One of the major barriers in transformation of microalgae is how to obtain decent expression of the transgene in microlagae. At the transcriptional level, regulatory elements contained in the expression cassette are important for correct and efficient gene expression. Because promoters available for microalgae are currently limited, promoter sequences from one organism could give inadequate recognition in other organisms and result in low or no transcription. 5´ and 3´ UTRs are also crucial for gene expression because they provide specific recognition sites for correct RNA processing. A number of studies have shown that these UTR sequences are the key for polyadenylation, appropriate nuclear transport and RNA stability, which highly influence gene expression in eukaryotes (Cerutti *et al*., 1997; Lumbreras *et al*., 1998).

Even if a proper promoter and regulatory elements are included in the expression cassette to ensure transcription of a transgene, another major barrier is the codon bias used in each microalgal species. It has been demonstrated that microalgal genomes contain very high GC content, for example, 61% in *Chlamydomonas* and 71% in *Monoraphidium* (Jarvis *et al*., 1992). This suggests a high variation in codon usage compared to other organisms. The limitation of tRNAs that correspond to certain codons in a specific microalgal species could result in low or no gene expression when expressing heterologous genes obtained from different organisms. Codon optimization has been shown to be the key to improve gene expression. Codon optimization of GFP for *Chlamydomonas* showed that the expression level was increased five folds (Fuhrmann *et al*., 1999).

Another major problem without solutions for now is the instability transgene expression. A number of previous studies have shown that the transgene expression was suppressed after maintaining in non-selective media for a length of time. The silencing effects could be due to various aspects of epigenetic

controls that are related to the response of virus invasion, transposable element or transgene found in eukaryotic system. This prevents the practicality of growing transgenic microalgae in a large scale or an open pond, because the addition of the antibiotic in a large-scale culture is not cost-effective and expression of the transgene in transgenic cells will gradually be suppressed or eliminated at later stages (Cerutti *et al*., 1997; Wu-Scharf *et al*., 2000).

CONCLUSION

Microalgae have been nominated as the most promising source for bioenergy in the near future following various beneficial aspects over other sources. However, our current platforms for biodiesel production from microalgae are still far behind those of petroleum based fuels in terms of cost effectiveness and production scale. Inevitably, the performance of the microalgae strain holds the essential key for the improvement of the platform, and evidently, with our knowledge, we cannot solely rely on the microalgal strains obtained from natural sources. Genetic manipulations are the vital key for the improvement of the microalgal strains. Although the progress in gene manipulation in microalgal system was slow in the past, the availability of functional/putative genes and genome sequences is providing a big leap in strain improvement. Various strains isolated from nature possessing each of the key traits can be used as bases for further improvements and their traits could be combined into one algal strain so called the “ideal strain” in the future.

REFERENCES

Anila, N., Chandrashekar, A., Ravishankar, G.A. and Sarada, R. 2011. Establishment of *Agrobacterium tumefaciens*-mediated genetic transformation in *Dunaliella bardawil. Eur J Phycol* 46: 36–44.

Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., *et al*. 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306: 79–86.

Banerjee, A., Sharma, R., Chisti, Y. and Banerjee, U.C. 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. *Crit Rev Biotechnol* 22: 245–279.

Baroli, I., Do, A.D., Yamane, T. and Niyogi, K.K. 2003. Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects *Chlamydomonas reinhardtii* from photooxidative Stress. *Plant cell* 15: 992–1008.

Beer, L.L., Boyd, E.S., Peters, J.W. and Posewitz, M.C. 2009. Engineering algae for biohydrogen and biofuel production. *Curr Opin Biotechnol* 20: 264–271.

Benemann, J.R. and Oswald, W.J. 1996. Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. Final Report to the USA Department of Energy. University of

California, Berkeley, California.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J., Salamov, A., Terry, A., Yamada, T., Dunigan, D.D., Grigoriev, I.V., Claverie, J.M. and Van Etten, J.L. 2010. The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. *Plant cell* 22: 2943–2955.

Borowitzka, M.A. 1992. Algal biotechnology products and processes-matching science and economics. *J Appl Phycol* 4: 267–279.

Borowitzka, M.A. 1997. Microalgae for aquaculture : Opportunities and constraints. *J Appl Phycol* 9: 393–401.

Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B. and Sanford, J.C. 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* 240: 1534–1537.

Boynton, J.E. and Gillham, N.W. 1993. Chloroplast transformation in *Chlamydomonas*. *Methods Enzymol* 217: 510–536.

Brown, L.E., Sprecher, S.L. and Keller, L.R. 1991. Introduction of exogenous DNA into *Chlamydomonas reinhardtii* by electroporation. *Mol Cell Biol* 11: 2328–2332.

Cassman, K.G. and Liska, A.J. 2007. Food and fuel for all : realistic or foolish? *Biofuels Bioprod Bioref* 1: 18–23.

Cerutti, H., Johnson, A.M., Gillham, N.W. and Boynton, J.E. 1997. Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. *Plant Cell* 9: 925–945.

Chassy, B.M., Mercenier, A. and Flickinger, J. 1988. Transformation of bacteria by electroporation. *Trends Biotechnol* 6: 303–309.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol Adv* 25: 294–306.

Chisti, Y. 2008. Biodiesel from microalgae beats bioethanol. *Trends Biotechnol* 26: 126–131.

Coll, J.M. 2006.Methodologies for transferring DNA into eukaryotic microalgae. *Span J Agric Res* 4: 316–330.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J. and Eijersbergen, A.G.M. 1998. *Agrobacterium tumefaciens* -mediated transformation of filamentous fungi. *Nat Biotechnol* 16: 839–842.

Davison, P.A., Hunter, C.N. and Horton, P. 2002. Overexpression of beta-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. *Nature* 418: 203–206.

Dehesh, K., Tai, H., Edwards, P., Byrne, J. and Jaworski, J.G. 2001. Overexpression of 3-ketoacyl-acyl-carrier protein synthase IIIs in plants reduces the rate of lipid synthesis. *Plant Physiol*. 125: 1103–1114.

Dent, R.M., Haglund, C.M., Chin, B.L., Kobayashi, M.C. and Niyogi, K.K. 2005. Functional genomics of eukaryotic photosynthesis using insertional mutagenesis

of *Chlamydomonas reinhardtii*. *Plant Physiol* 137: 545–556.

Derelle, E., Ferraz, C., Rombauts, S., Rouze, P., Worden, A.Z., Robbens, S., *et al*. 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc Natl Acad Sci USA* 103: 11647–11652.

Dunahay, T.G. 1993. Transformation of *Chlamy domonas reinhardtii* with silicon carbide whiskers. *Biotechniques* 15: 452–460.

Dunahay, T.G., Jarvls, E.E., Dais, S.S. and Roessler, P.G. 1996. Manipulation of microalgal lipid production using genetic engineering. *Appl Biochem Biotechnol* 57: 223–231.

El-Sheekh, M.M. 1999. Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. *Biol Plant* 42: 209–216.

Ferrante, P., Catalanotti, C., Bonente, G. and Giuliano, G. 2008. An optimized, chemically regulated gene expression system for *Chlamydomonas*. *PLoS ONE* 3: e3200.

Fischer, N. and Rochaix, J.D. 2001. The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii. Mol Genet Genomics* 265: 888–894.

Fuhrmann, M., Oertel, W. and Hegemann, P. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant J* 19: 353–361.

Gietz, D. and R. Woods. 2001. Genetic transformation of yeast. *Biotechniques* 30: 816–831.

González-Ballester, D., de Montaigu, A., Galván, A. and Fernández, E. 2005. Restriction enzyme site-directed amplification PCR: A tool to identify regions flanking a marker DNA. *Anal Biochem* 340: 330–335.

Grobbelaar, J.U. 2000. Physiological and technological considerations for optimising mass algal cultures. *J Appl Phycol* 12: 201–206.

Grossman, A. 2005. Paths toward algal genomics. *Plant Physiol* 137: 410–427.

Hall, L.M., Taylor, K. and Jones, D.D. 1993. Expression of a foreign gene in *Chlamydomonas reinhardtii*. *Gene* 124: 75–81.

Hanikenne, M., Krämer, U., Demoulin, V., Baurain, D. 2005. A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschizon merolae*. *Plant Physiol* 137: 428–446.

Jako, C., Kumar, A., Wei, Y., Zou, J., Barton, D.L., Giblin, E.M., Covello, P.S. and Taylor, D.C. 2001. Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerolacyltransferase enhances seed oil content and seed weight. *Plant Physiol* 126: 861–874.

Jarvis, E.E., Dunahay, T.G. and Brown, L.M. 1992. DNA nucleoside composition and methylation in several species of microalgae. *J Phycol* 28: 356–362.

Kathir, P., Lavoie, M., Brazelton, W.J., Haas, A., Lefebvre, P.A. and Silflow, C.D. 2003. Molecular map of the *Chlamydomonas reinhardtii* nuclear genome molecular. *Eukaryot Cell* 2: 362–379.

Kathiresan, S., Chandrashekar, A., Ravishankar, G.A. and Sarada, R., 2009. A*grobacterium*-mediated transformation in the green alga *Haematococcus pluvialis* (Chlorophyceae, Volvocales). *J Phycol* 45: 642–649.

Kilian, O., Benemann, C.S.E., Niyogi, K.K. and Vick, B. 2011. High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proc Natl Acad Sci USA* 108: 21265–21269.

Kindle, K.L. 1990. High frequency nuclear transformation of *Chlamydomonas reinhardtii. Proc Natl Acad Sci USA* 88: 1228–1232.

Kindle, K.L. 1998. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Methods Enzymol* 297: 27–38.

Kindle, K.L., Richards, K.L. and Stern, D.B. 1991. Engineering the chloroplasts genome: New techniques and capabilities for chloroplast transformation in *Chlamydo monas reinhardtii*. *Proc Natl Acad Sci USA* 88: 1721–1725.

Kumar, S.V., Misquitta, R.W., Reddy, V.S., Rao, B.J. and Rajam, M.V. 2004. Genetic transformation of the green alga-*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. *Plant Sci* 166: 731–738.

Kunik, T., Tzfira, T., Kapulnik, Y., Gafni, Y., Dingwall, C. and Citovsky, V. 2001. Genetic transformation of HeLa cells by Agrobacterium. *Proc Natl Acad Sci USA* 98: 1871–1876.

La Russa, M., Bogen, C., Uhmeyer, A., Doebbe, A., Filippone, E., Kruse, O. and Mussgnug, J.H. 2012. Functional analysis of three type-2 *DGAT* homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. *J Biotechnol* 162: 13–20.

Le Martret, B., Poage, M., Shiel, K., Nugent, G.D. and Dix, P.J. 2011. Tobacco chloroplast transformants expressing genes encoding dehydroascorbate reductase, glutathione reductase, and glutathione-S-transferase, exhibit altered anti-oxidant metabolism and improved abiotic stress tolerance. *Plant Biotechnol J* 9: 661–673.

Ledford, H.K. and Niyogi, K.K. 2005. Singlet oxygen and photo-oxidative stress management in plants and algae. *Plant Cell Environ* 28: 1037–1045.

Leon-Banares, R., Gonzalez-Ballester, D., Galvan, A. and Fernandez, E. 2004. Transgenic microalgae as green cell-factories. *Trends Biotechnol* 22: 45–52.

Li, Z., Keasling, J.D. and Niyogi, K.K. 2012. Overlapping photoprotective function of vitamin E and carotenoids in *Chlamydomonas*. *Plant Physiol* 158: 313–323.

Lohuis, M.R. and Miller, D.J. 1998. Genetic transformation of dinoflagellates (*Amphidinium* and *Symbiodinium*): expression of GUS in microalgae using heterologous promoter constructs. *Plant J* 13: 427–435.

Lü, J., Sheahan, C. and Fu, P. 2011. Metabolic

engineering of algae for fourth generation biofuels production. *Energy Environ Sci* 4: 2451–2466.

Lumbreras, V., Stevens, D.R. and Purton, S. 1998. Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J* 14: 441–447.

Mata, T.M., Martins, A.A. and Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: *A review Renew Sust Energ Rev* 14: 217–232.

Matsuzaki, M., Misumi, O., Shin-I, T., Maruyama, S., Takahara, M., Miyagishima, S.Y., *et al*. 2004. Genome sequence of the ultra small unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature* 428: 653–657.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., *et al*. 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* 318: 245–251.

Michinaka, Y., Shimauchi, T., Aki, T., Nakajima, T., Kawamoto, S., Shigeta, S., Suzuki, O. and Ono, K. 2003. Extracellular secretion of free fatty acids by disruption of a fatty acyl-CoA synthetase gene in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 95: 435–440.

Miller, R., Wu, G.X., Deshpande, R.R., Vieler, A., Gärtner K., Li, X.B., Moellering, E.R., Zäuner, S., Cornish, A.J., Liu, B., Bullard, B., Sears, B.B., Kuo, M.H., Hegg, E.L., Shachar-Hill, Y., Shiu, S.H. and Benning, C. 2010. Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. *Plant Physiol* 154: 1737–1752.

Mirón, A.S., Gómez, A.C., Camacho, F.G., Grima, E.M. and Chisti, Y. 1999. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. *J. Biotechnol.* 70*:* 249–270.

Nagl, W. 1978. *Endopolyploidy and Polyteny in Differentiation and Evolution*. North-Holland Publishing Company, Amsterdam.

Nakamoto, H., Suzuki, N. and Roy, S.K. 2000. Constitutive expression of a small heat-shock protein confers cellular thermotolerance and thermal protection to the photosynthetic apparatus in cyanobacteria. *FEBS Lett* 483: 169–174.

Pighin, J.A., Zheng, H., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L. and Samuel, A.L. 2004. Plant cuticular lipid export requires an ABC transporter. *Science* 306: 702–704.

Polle, J.E., Benemann, J.R., Tanaka, A. and Melis, A. 2000. Photosynthetic apparatus organization and function in the wild type and a chlorophyll *b-less* mutant of *Chlamydomonas reinhardtii* dependence on carbon source. *Planta* 211: 335–344.

Potvin, G. and Zhang, Z. 2010. Strategies for high-level recombination protein expression in transgenic microalgae: A review. *Biotechnol Adv* 28: 910–918.

Poulsen, N. and Kroger, N. 2005. A new molecular tool for transgenic diatom: control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette.

FEBS J 272: 3413–3423.

Radakovits, R., Jinkerson, R.E., Darzins, A. and Posewitz, M.C. 2010. Genetic engineering of algae for enhanced biofuel production. *Eukaryot Cell* 9: 486–501.

Ramachandra, T.V., Mahapatra, D.M., Karthick, B. and Gordon, R. 2009. Milking diatoms for sustainable energy : Biochemical engineering versus gasoline-secreting diatom solar panels. *Renew Energ* 48: 8769–8788.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K. and Peccia, J. 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: pathway description and gene discovery for production of next-generation biofuels. *BMC Genomics* 12: 148.

Rochaix, J. D. and van Dillewijn, J. 1982. Transformation of the green alga *Chlamydomonas reinhardtii* with yeast DNA. *Nature* 296: 70–72.

Rodolfi, L., ChiniZittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G. and Tredici, M.R. 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol Bioeng* 102: 100–112.

Rohr, J., Sarkar, N., Balenger, S., Jeong, B.-R. and Cerutti, H. 2004. Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*. *Plant J* 40: 611–621.

Rubio, F.C., Camacho, F.G., Sevilla, J.M., Chisti, Y. and Grima, E.A. 2003. A mechanistic model of photosynthesis in microalgae. *Biotechnol Bioeng* 81: 459–473.

Rymarquis, L.A., Handley, J.M., Thomas, M. and Stern, D.B. 2005. Beyond Complementation. Map-Based Cloning in *Chlamydomonas reinhardtii*. *Plant Physiol* 137: 557–566.

Schiedlmeier, B., Schmitt, R., Muller, W., Kirk, M.M., Gruber, H., Mages, W. and Kirk, D. L. 1994. Nuclear transformation of *Volvox carteri*. *Proc Natl Acad Sci USA* 91: 5080–5084.

Schroda, M., Vallon, O., Wollman, F.A. and Beck, C.F. 1999. A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant cell* 11: 1165–1178.

Schulz, B. and Kolukisaoglu, H.U. 2006. Genomics of plant ABC transporters: the alphabet of photosynthetic life forms or just holes in membranes? *FEBS Lett* 580: 1010–1016.

Sizova, I., Fuhrmann, M. and Hegemann, P. 2001. A *Streptomyces rimosusaph VIII* gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. *Gene* 277: 221–229.

Shimogawara, K., Fujiwara, S., Grossman, A. and Usuda, H. 1998. High-efficency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics* 148: 1821–1828.

Shrager, J., Hauser, C., Chang, C. W., Harris, E. H., Davies, J., McDermott, J., Tamse, R., Zhang, Z. and Grossman, A.R. 2003.

Chlamydomonas reinhardtii genome project. A guide to the generation and use of the cDNA information. *Plant Physiol* 131: 401–408.

Stevens, D.R. and Purton, S. 1996. The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. *Mol Gen Genet* 251: 23–30.

Sugimoto-Shirasu, K. and Roberts, K. 2003. "Big it up": endoreduplication and cell-size control in plants. *Curr Opin Plant Biol* 6: 544–553.

Sun, X., Meng, X., Xu, Z. and Song, R. 2010. Expression of the 26S proteasome subunit RPN10 is upregulated by salt stress in *Dunaliella viridis*. *J Plant Phys* 167: 1003–1008.

Tam, L.Y. and Lefebvre, P.A. 1993. Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. *Genetics* 135: 375–384.

Teng, C., Qin, S., Liu, J., Yu, D., Liang, C. and Tseng, C. 2002. Transient expression of *lacZ* in bombarded unicellular green alga *Haematococcus pluvialis*. *J Appl Phycol* 14: 495–500.

Tetali, S.D., Mitra, M. and Melis, A. 2007. Development of the light-harvesting chlorophyll antenna in the green alga *Chlamydomonas reinhardtii* is regulated by the novel *Tla1* gene. *Planta* 225: 813–829.

Töpfer, R., Martini, N. and Schell, J. 1995. Modification of plant lipid synthesis. *Science* 268: 681–686.

Verstrepen, K.J., Derdelinckx, G., Verachtert, H. and Delvaux, F.R. 2003. Yeast flocculation: what brewers should know. *Appl Microbiol Biotechnol* 61: 197–205.

Wang, C., Wang, Y., Su, Q. and Gao, X. 2007. Transcient expression of the GUS gene in a unicellular marine alga, *Chlorella* sp. MACC/C95, via electroporation. *Biotechnol Bioprocess Eng* 12: 180–183

Walbot, V. 1992. Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. *Annu Rev Plant Physiol Plant Mol Biol* 43: 49–78.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S. and Goodenough, U. 2009. Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot Cell* 8: 1856–1868.

Wijffels, R.H. and Barbosa, M.J. 2010. An outlook on microalgal biofuels. *Science* 329: 796-799.

Wu-Scharf, D., Jeong, B., Zhang, C. and Cerutti, H. 2000. Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-Box RNA helicase. *Science* 290: 1159–1163.

Zhao, T., Wang, W., Bai, X. and Qi, Y. 2009. Gene silencing by artificial microRNAs in *Chlamydomonas*. *Plant J* 58: 157–164.

Zittelli, G.C., Rodolfi L., Biondi, N. and Tredici, M.R. 2006. Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. *Aquaculture* 261: 932–943.