Biotechnology Advances 30 (2012) 1602–1613

Contents lists available at SciVerse ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Advances in genetic engineering of marine algae

Song Qin [a,*,1], Hanzhi Lin [b,c,1], Peng Jiang [b]

[a] Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, Shandong, China
[b] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, Shandong, China
[c] Graduate University of Chinese Academy of Sciences, Beijing 100049, Beijing, China

ARTICLE INFO

Available online 24 May 2012

Keywords:
Genetic engineering
Transgenic algae
Marine algae
Genomics
Vector
Expression system
Photobioreactor
Biosafety

ABSTRACT

Algae are a component of bait sources for animal aquaculture, and they produce abundant valuable compounds for the chemical industry and human health. With today's fast growing demand for algae biofuels and the profitable market for cosmetics and pharmaceuticals made from algal natural products, the genetic engineering of marine algae has been attracting increasing attention as a crucial systemic technology to address the challenge of the biomass feedstock supply for sustainable industrial applications and to modify the metabolic pathway for the more efficient production of high-value products. Nevertheless, to date, only a few marine algae species can be genetically manipulated. In this article, an updated account of the research progress in marine algal genomics is presented along with methods for transformation. In addition, vector construction and gene selection strategies are reviewed. Meanwhile, a review on the progress of bioreactor technologies for marine algae culture is also revisited.

Contents

* Corresponding author at: No. 17 Chunhui Road, Laishan District, Yantai 264003, Shandong Province, China. Tel.: +86 535 2109005.
E-mail addresses: sqiniocas@gmail.com, sqin@yic.ac.cn (S. Qin).
[1] These authors contributed equally to this work.

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doi:10.1016/j.biotechadv.2012.05.004

1. Introduction

Marine algae, including marine cyanobacteria, marine eukaryotic microalgae, and seaweed, are the oldest members of the plant kingdom, dating back three billion years and distributing from the polar region to tropical areas and from nutrient-rich coastal seas to oligotrophic open oceans. They are photoautotrophs unified primarily by their lack of roots, leaves, and other organs that characterize higher plants and their possession of Chlorophyll *a*. Marine algae range in size from microscopic individual cells of microalgae to huge plants that are greater than 30 m long and are called macroalgae *Macrocystis*. Marine algae are responsible for approximately 40%–50% of the photosynthesis that occurs on Earth each year (Falkowski et al., 1998). Marine photosynthesis is dominated by algae, including cyanobacteria (i.e., blue-green algae or cyanophyta) and eukaryotic taxa. Marine algae are a component of the bait sources for animal aquaculture and produce abundant valuable compounds for the chemical industry and human health, including oils (e.g., triglyceride), polysaccharides (e.g., algin, agar), pigments (e.g., phycobiliproteins, carotenoids), and also potential new pharmaceuticals (Apt and Behrens, 1999; Chisti, 2007, Lin et al., 2011, Witvrouw and DeClercq, 1997). Recently, a microbial platform that can simultaneously degrade, uptake, and metabolize alginate was established based on new discovered enzymes for alginate transport and metabolis, which enables bioethanol production directly from brown macroalgae via a consolidated process (Wargacki et al., 2012). The ancient cyanobateria are the last common ancestor of all oxygenic photosynthetic lineages, which have the closet evolutionary relationship with heliobacteria and other anaerobic photoautotrophs (Xiong et al., 2000), while photosynthetic eukaryotes acquired their photosynthetic properties from endosymbiosis with cyanobacteria (Gray, 1992; Reyes-Prieto and Bhattacharya, 2007). The green algae are primitive members of the kingdom Plantae from which land plants evolved approximately 500 million years ago (Parker et al., 2008, Wise, 2006). Due to the algal complex and the unique genetic and evolutionary scheme, the genetic engineering of algae must be considered to apply both of the methodologies from prokaryotic microorganisms and plants. Since the end of the 1970s, marine algal genetic engineering began being implemented in the model system cyanobacterial strain *Synechococcus* 7002, which can be transformed by exogenously adding homologous DNA carrying a selectable marker (Buzby et al., 1985; Matsunaga and Takeyama, 1995; Stevens and Porter, 1980). In the late 1980s, several eukaryotic marine microalgae and seaweeds were successfully transformed by different transformation methods, e.g., microinjection in the marine macro-green alga *Acetabalaria* sp. (Neuhaus et al., 1986), plasmid vectors in the marine diatom *Cyclotella cryptica* (Dunahay et al., 1995), and gene gun or the biolistic method in the macro-red alga *Eucheuma* sp. (Kurtzman and Cheney, 1991) and brown alga *Laminaria japonica* (Qin et al., 1998). Entering the new century, the trend of the genetic engineering of marine algae has been to apply the transgenic marine algae as cell factories and marine bioreactors (León-Bañares et al., 2004; Qin et al., 2005; Zaslavskaia et al., 2001). To date, the most successful algal genetic transformation system is still the model system of the eukaryotic freshwater green alga *Chlamydomonas reinhardtii* (Grossman, 2000) whose nucleus and chloroplast transformations have reached promising commercial relevance (Franklin and Mayfield, 2004, 2005). Although *C. reinhardtii* may not be a suitable applied species, the technologies established with this species have the potential for applications in other algal species (Hannon et al., 2010).

With today's rapidly growing demand and development for bioenergy from algae and the profitable market for cosmetics and pharmaceuticals from algal natural products, the genetic engineering of marine algae has been attracting an increasing amount of attention as a crucial systemic technology to overcome the biomass problem in industrial applications (John et al., 2011), to modify the metabolic pathway for high-value products (Schmidt et al., 2010), and to engineer the bio-bricks and design the artificial photoautotroph in the rising and promising field of synthetic biology (Heidorn et al., 2011, Muers, 2012). Nevertheless, to date, only a few marine algae species have been genetically manipulated successfully. In this article, an updated account of the research progress in marine algal genomics is presented as well as methods for transformation. In addition, vector construction and gene selection strategies are reviewed. Meanwhile, a review of the progress of bioreactor technologies for marine algae culture is also revisited.

2. Marine algal genomics

Genomes are fundamental for genetic manipulation and further genetic engineering, which not only provide the location and the distribution of metabolic pathways and enzymes but also aid in the identification of elements that can improve genetic engineering, including cis-acting elements, trans-acting factors, and other regulatory elements.

2.1. Cyanobacterial genomics

In evolutionary terms, chloroplasts are cyanobacteria (Allen et al., 2011). The emerging field of marine algal genomics first began with publications of the three genomes of the smallest known oxygen-evolving autotroph *Prochlorococcus* (Dufresne et al., 2003, Rocap et al., 2003). To date, over 20 cyanobacterial genomes have been released. Marine cyanobacteria possess several traits in their genome that are different from other algae. Examples of these differences include their out-membrane light harvesting antenna, a two-component signal transduction system, and their autotrophic metabolism. Many cyanobacteria have water-soluble, light-harvesting protein-pigment complex phycobilisomes, which can reach a width of 40 nm (Yi et al., 2005) and attach to the cytoplasmic surface of the thylakoid membrane. These pigments (chromophores) are phycobilins as opposed to chlorophylls and covalently bind to their apoproteins. In marine *Synechococcus* spp, the genes for the metabolism of phycobiliproteins are concentratedly distributed in several operons or gene clusters. The cyanobacteria can intimately attune to ambient light conditions with shifts in the levels of their phycobilisome composition, i.e., chromatic acclimation (Kehoe and Gutu, 2006), which can be achieved by the regulations of a two-component signal system (Gutu and Kehoe, 2012). However, in *Prochlorococcus*, most of the genes for phycobiliproteins disappear, and only a small set of genes for phycoerythrin Type III and their reductases is conserved in the genome, which suggests that the genes for phycobilierythrin are being lost through selection in the evolutionary process (Ting et al., 2001). Compared to the complicated environments

of freshwater and soil, the marine environment is relatively stable; thus, marine cyanobacteria may not require an entire, large set of the two-component (*hik* & *rer* genes) signal system. Most genomes of the marine cyanobacteria that are currently available harbor a very limited repertoire of *hik* and *rer* genes, including only five to six potential *hik* genes and seven to 11 potential *Rer* genes, as compared to the 13 to 95 potential *hik*s and 23 to 94 potential *Rer*s of freshwater and terrestrial strains (Ashby and Houmard, 2006; Mary and Vaulot, 2003). In the N metabolism of the assimilation of ammonium into organic N compounds, as in other cyanobacteria, marine *Synechococcus* and *Prochlorococcus* strains use the glutamine synthetase glutamate synthase pathway to assimilate ammonium (Scanlan et al., 2009). However, several *Prochlorococcus* genomes do not contain genes for the transport systems of nitrate, nitrite, cyanate, and urea, which are present in freshwater cyanobacteria. They also do not contain the coding for a nitrate/nitrite permease that was recently discovered in a marine *Synechococcus* (Dufresne et al., 2003; El Alaoui et al., 2001), which may be the most surprising discovery regarding substrate utilization (Scanlan et al., 2009).

2.2. Eukaryotic algal genomics

The emerging field of marine eukaryotic algal genomics first began with the discovery of the complete sequences for all three remnant nucleomorph chromosomes of the cryptomonad *Guillardia theta* (Douglas et al., 2001). Since then, numerous genomes have been sequenced or are in progress for many marine algae. The draft genome and transcriptome data from red alga *Cyanophora paradoxa* which provides evidence for a single origin of the primary plastid in the Plantae were sequenced and analyzed showing that this basally diverging algal genome retains ancestral features of starch biosynthesis, fermentation, and plastid protein translocation common to plants and algae but lacks typical eukaryotic light-harvesting complex proteins (Price et al., 2012). The eukaryotic algal genomes were mainly shaped by the forces of endosymbiotic gene transfer and lateral gene transfer. Eukaryotic photoautotrophs appeared at least 1.2 billion years ago when a nonphotosynthetic unicellular eukaryote successfully took in a cyanobacterium that resulted in a two-membrane photosynthetic plastid containing a cyanobacterial-derived genome (Reyes-Prieto and Bhattacharya, 2007). The analyses according to a plastid multi-gene phylogeny with Bayesian and maximum likelihood phylogenic methods supports an ancient origin of photosynthetic eukaryotes with the primary endosymbiosis sometime before 1.558 billion years ago (Yoon et al., 2004). This primary endosymbiotic event is hypothesized to have produced three photosynthetic lineages: a green lineage (Chlorophyll *a* and *b*), and the other red and glaucophyte lineages (chlorophyll *a* and phycobilins) (Parker et al., 2008). The split of the red and green algae is calculated to have occurred about 1.5 billion years ago (Yoon et al., 2004). Secondary endosymbiotic events generated further diversity among photosynthetic eukaryotes. Dominant marine eukaryotic algae (diatoms, dinoflagellates, haptophytes, cryptomonads) were derived from a secondary endosymbiosis possessing plastids from the invasion of a red alga. The secondary endosymbiosis is calculated to have occurred about 1.3 billion years ago (Yoon et al., 2004). Tertiary endosymbiosis created a greater diversity within the dinoflagellates. Taxa, which were derived from secondary or tertiary endosymbiosis, obtained the plastid genome by retaining the engulfed photosynthetic eukaryote while the engulfed nucleus genome was either lost (including transferring several genes to the host nuclear) or greatly reduced. The plastid genomes vary broadly in size and gene content (Green, 2011, Grzebyk et al., 2003), and for several peridinin-containing dinoflagellates, the plastid genomes have substantially decreased to one or two genes or even none at all (Zhang et al., 1999). Cryptomonads (containing a red algal plastid) and Chlorarachniophytes (green algal plastid) coordinate four genomes (two nuclear and one each from the plastid or the mitochondria) (Gilson et al., 2006, Tanifuji et al., 2011). In the remnant nucleomorph genome of Cryptomonad *Guillardia theta*, half of the genes code for unknown function proteins (Douglas et al., 2001). Both the cryptophyte and chlorarachniophyte nucleomorph genomes display high gene densities, and only a few of the genes are involved in plastid functions.

Lateral gene transfer (LGT) occurs independently of an endosymbiotic event. The first elegant work describing the full impact of LGT on photosynthetic eukaryotics was completed in the chlorarachniophyte. The plastid target genes that account for 21% of the nucleus genome of the chlorarachniophyte, *Bigelowiella natans* were acquired by LGT from numerous other sources: streptophyte algae, red algae (or algae with red algal endosymbionts), as well as bacteria (Archibald et al., 2003). To date, an increasing number of phylogenetic works found that this mechanism plays a key role in the photosynthetic eukaryotic gene and in genomic evolution (Chan et al., 2011; Keeling and Palmer, 2008). Adjacent genes transferred from cyanobateria to eukaryotic dinoflagellates by LGT could acquire a new fusion plastid-targeting peptide (Waller et al., 2006). In the genome of *Ostreococcus tauri*, one chromosome was postulated to be from a different origin than the remainder of the genome according to the G + C content, the codon usage, the plentiful amount of transposable elements, and a phylogenetic analysis of peptide-encoding genes (Derelle et al., 2006). Viruses could mediate the LGT, especially in a marine environment by transduction. It is acknowledged that the virus-mediated LGT could occur between cyanobacteria and cyanophages (Sullivan et al., 2006), and between viruses and eukaryotic algae, such as brown seaweed (Cock et al., 2010; Delaroque et al., 2001) and diatoms (Montsant et al., 2007).

3. Methods for transformation in marine algae

To date, more than 20 different strains of marine algae have been transformed successfully with various transformation methods (Table 1). In some cases, transformation resulted in the successful stable expression of transgenes from either the nucleus or the plastid, but in many cases, only transient expression was observed.

3.1. Trans-conjugation

Trans-conjugation is the transfer of DNA between a cyanobacterial cell and a bacterial cell (usually *Escherichia coli*) by direct cell-to-cell contact or by a bridge-like connection between two cells. Although this method is more frequently used in the freshwater cyanobacterial genetic manipulation, the versatility of gene transfer by trans-conjugation in marine cyanobacteria was first demonstrated in five strains of marine *Synechococcus*, one strain of marine *Synechocystis* and one strain of marine *Pseudanabaena* (Sode et al., 1992). Conjugation was implemented using a mobilizable transposon and a broad-host-range vector pKT230 (IncQ). This research confirmed the wide applicability of conjugation in marine cyanobacteria. Three typical marine *Synechococcus*, WH7803 isolated from shelf waters and WH8102 and WH8103 representative of open-ocean environments, were successfully genetically manipulated by a conjugation method to introduce both a replicative vector and a suicide vector (Brahamsha, 1996). A plasmid DNA containing green fluorescent protein (GFP) was transferred into a *Prochlorococcus* strain by interspecific conjugation with *E. coli*, and the expression of this protein was detected by Western blotting and cellular fluorescence (Tolonen et al., 2006). This is the first report of GFP expression in oceanic cyanobacteria.

3.2. Natural transformation and induced transformation

Natural transformation and induced transformation could allow a cyanobacterial cell to absorb extrinsic DNA directly in the form of natural competence cells or be artificially induced to competence cells whenever they are undergoing exponential growth. In marine cyanobacteria,

Table 1
Main characteristics of transformation methods used in marine algae genetic engineering.

Methods	Characteristics
Trans-conjugation	It is mainly in cyanobacteria and rarely used at present.
Natural transformation and induced transformation	It is mainly in cyanobacteria and rarely used at present.
Electroporation	It has simple procedure, and is used universally to different genera but constrained in brown algae.
Biolistic transformation	Exogenous DNA can be introduced into various cells and tissues. Diversified vectors can be applied to overcome the genetic background insufficiency of the substances. The manipulation is controllable and mature. But it requires specialized and high cost equipment.
Glass beads	The procedure is simple and it doesn't need high cost transgenic equipment. But it is constrained in macroalgae due to immature protoplast regeneration technology.
Silicon carbon whiskers method	It overcomes the cell wall's obstruction of exogenous DNA compared to glass beads method and it has inexpensive cost. But it requires strict safeguard to avoid the inhalation hazard.
Microinjection	Whereas it is a highly efficient and low cost method but it has complicated and delicate procedure.
Artificial transposon method	Exogenous gene could be directionally integrated into receptor's genome.
Recombinant eukaryotic algal viruses	It has potential application in brown algae but still needs extensive and comprehensive fundamental studies.
Agrobacterium tumefaciens-mediated genetic transformation	The efficiency is highly dependent on many elements and this method is technically challenging.

natural transformation has been reported only for *Synechococcus* sp. PCC7002 (Buzby et al., 1985) to date, while others are mainly found in freshwater strains. Some marine cyanobacteria could be transformed by competent cycle determination. By treating with ethidium bromide, a cured strain marine *Synechococcus* sp. NKBG042902-YG 1116 successfully transformed under dark incubation conditions for 16 h (Matsunaga and Takeyama, 1995). The mechanism of competence in marine cyanobacteria may be similar to that in bacteria (Porter, 1986). However, the transformation efficiencies of the marine *Synechococcus* strains were ten times lower than those for the freshwater *Synechococcus* strains. Polysaccharides may prevent DNA uptake, which were found surrounding the marine *Synechococcus* cell. Thus, due to the requisition of simple, quick and highly efficient transformation methods, these two transformations were rarely used and gradually replaced with electroporation when taking into account marine cyanobacterial genetic transformation.

3.3. Electroporation

Gene transfer by electroporation has been applied in various cells and bacteria for over 30 years because of the simplicity of the procedure and a high efficiency with a small amount of DNA (Neumann et al., 1982, Zimmermann et al., 1975). Electroporation can transfer extrinsic genes independent of the cell's ability and universally to different genera. The first usage in marine cyanobacterium was performed in marine unicellular *Synechococcus* sp. NKBG042902-YG 1116 (Matsunaga et al., 1990). The electric field strength for marine cyanobateria was lower than that for fresh water strains. The decrease in efficiency due to the electric field strength can be compensated for by enhancing the $CaCl_2$ pretreatment of marine strains. Efficient electroporation-mediated transformation was achieved in both wild-type and cell wall-deficient eukaryotic *Chlamydomonas reinhardtti* strains (Brown et al., 1991). The efficiency of electroporation was two orders of magnitude higher than that obtained with the glass beads method to introduce exogenous DNA to algal cells (Shimogawara et al., 1998). To date, the electroporation transformation was established in many marine genera from prokaryotic cells to eukaryotic red algae, green algae and diatoms. Recently, the "star" marine alga for potential biofuel production, known as the *Nannochloropsis* sp., was successfully genetically transformed by electroporation, and several genes involved in the nitrogen metabolism were knockout genes by this homologous recombination method (Kilian et al., 2011). This technology will rapidly advance subsequent functional genomic research (Pan et al., 2011) and the application in biotechnologies, particularly the R&D of marine algal bio-energy. However, the application of the electroporation technology in brown algae is constrained by undeveloped protoplast preparation and regeneration technologies.

3.4. Biolistic transformation

Direct gene transfer by the biolistic method (micro-particle bombardment) has been proven to be the most efficient method and is highly reproducible in introducing exogenous DNA into algal cells. This method has been successfully employed for the transformation of many microalgal nuclear and chloroplast expression systems, and it is not surprising that biolistic transformation remains the most useful tool for transgenic studies of marine macroalgae regardless of their cell walls and life cycle. The biolistic method has the following advantages: ① exogenous DNA can be introduced into various cells and tissues, including plants, animals, microbes, and even pollen and other peculiar acceptors. It is the only effective method that can repeatedly transform chloroplasts, mitochondria and other organelles. ② Diversified vectors can be applied in biolistic transformation. Due to the limited understanding of the structure and function of the genetic substances of most algae, it is sometimes difficult to construct useful algal endogenous vectors. The vectors from *E. coli* were usually used in algal biolistic transformations. Theoretically speaking, the size of the plasmid from an *E. coli* is suitable for biolistic manipulation to prevent rupture and losing the plasmid. Furthermore, the developed and diverse vectors from high plants provide a repertory for future transgenic manipulation design. ③ Although particle bombardment requires specialized and high cost equipment (gene gun), the manipulation procedure of biolistic transformation is controllable and mature. Nearly all of the physical and chemical parameters (rupture pressure, DNA concentration, particle travel distances, and vacuum degree) in the gene gun can be adjusted to different algal materials and acceptors.

Marine cyanobacterium *Synechococcus* sp. NKBG15041c was transformed successfully using particle bombardment with bacterial magnetic particles (BMPs) purified from one magnetic bacterium known as *Magnetospirillum* sp. AMB-1. This particle is covered with a thin phospholipid layer and can bind larger quantities of DNA than gold or tungsten particles (Matsunaga et al., 1991). In addition, a more efficient transformation was obtained with BMPs than with gold particles. This experiment demonstrates the advantage of BMPs as a DNA carrier during biolistic processes for marine cyanobacteria.

To date, the available tools for genetically engineering marine diatoms remain sparse. Biolistic transformation is the only efficient tool to genetically manipulate marine diatoms. A stable nuclear transformation system was established and developed for several diatoms using particle bombardment in the 1990s (Apt et al., 1996; Dunahay et al., 1995; Falciatore et al., 1999). Recently, the utility of several selectable marker and reporter genes for use in *Phaeodactylum tricornutum* was examined (Zaslavskaia et al., 2000). Transformation methods with particle bombardment have been established for several species of diatoms, including the centric diatom *Thalassiosira pseudonana* (Poulsen et al., 2006), *Thalassiosira weissflogii* (Falciatore et al., 1999), *Chaetoceros* sp. (Miyagawa-Yamaguchi et al., 2011), *C. cryptica* (Dunahay et al., 1995), and the pinnate diatoms *Navicula saprophila* (Dunahay et al.,

1995), *P. tricornutum* (Apt et al., 1996, Falciatore et al., 1999, Miyagawa et al., 2009, Zaslavskaia et al., 2000), *Cylindrotheca fusiformis* (Fischer et al., 1999; Poulsen and Kröger, 2005).

Since 1992, biolistic methods have been applied to marine macroalgal genetic engineering (Cheney and Kurtzman, 1992). A transient transformation system has been established in the genera *Porphyra* (Kuang et al., 1998, Zhang et al., 2010) whose cultivation currently provides a yearly turnover of approximately 1×10^9 US dollars (Pulz and Gross, 2004) and forms a mature algal industry for food, cosmetics, and other high value products. The five types of acceptor cells (juvenile sporophytes, male and female gametophytes, tissue pieces from sporophytes, and parthenogenetic sporophytes) from brown macroalgae can all be transformed by particle bombardment (Jiang et al., 2003, Qin et al., 1999). Because bombardment does not affect the growth and development of female gametophytes, an expression system for *L. japonica* gametophytes could be successfully established (Qin et al., 2005) (expression system will be discussed in detail in Section 6).

3.5. Glass beads

Agitation with glass beads has been used to efficiently introduce foreign DNA into microalgae and was first reported in the freshwater alga *C. reinhardtii* (Kindle, 1990). The advantages of glass beads are their simplicity and independence from expensive and specialized equipment compared to biolistic transformation. The genetic transformation system for *Dunaliella salina*, which lacks a rigid cell wall, by glass beads has been successfully established, and the glass beads method is more efficient and repeatable, more easily controlled and less physically destructive to cells than electroporation and particle bombardment for the transformation of *D. salina* when these three methods were conducted and compared at the same time (Feng et al., 2009). The red seaweed *Porphyra haitanensis* was transformed with glass beads by agitating the freshly released conchospores, which have either thin cell walls or none at all. The maximum number of transformants was more than six out of the 1 million agitated conchospores (Wang et al., 2010). The main drawback of this method is its inability to transfer DNA into cells with thick cell walls (Coll, 2006). Cells with thick cell walls should be pre-treated to digest the walls with an enzyme to become protoplasts. Then, they can be mixed and agitated with the glass beads and the membrane fusion agent polyethylene glycol (PEG) during agitation. However, in some seaweeds, cell viability decreases when the cell walls are removed, and the development and the differentiation of callus tends to complicate the isolation of transformants (Polnefuller and Gibor, 1984). For the state of the art in macroalgal protoplast regenerations, the list of seaweed species capable of regenerating into complete plants from protoplasts remains limited; however, the list is steadily growing (Baweja et al., 2009, Reddy et al., 2008).

3.6. Silicon carbon whiskers method

The disadvantage of the glass beads method stated above is that it requires that a specific cellular and genetic trait (e.g., the cell wall deficient mutation or thin cell wall) in the host cells achieves efficient transformation or that the host cells form into wall-deficient cells by the use of an enzyme (e.g. autolysin). In contrast to agitation of the cells with glass beads, agitating the *C. reinhardtii* cells with silicon carbon (SiC) whiskers for up to 10 min results in a minor loss in cell viability. The SiC whisker method produced transformants at an efficiency of up to 10^{-5} per cell for walled cells and up to 10^{-4} per cell for cell wall deficient mutant strains (Dunahay, 1993). Because of its ability to overcome the cell wall's obstruction of exogenous DNA and its inexpensive cost, this technique has been attempted in the stable genetic transformation of marine dinoflagellates. The efficiency range was approximately 5–24 per 10^7 cells (ten Lohuis and Miller, 1998). Cell viability following SiC whisker agitation is greatly improved, but because of the unreliable source of SiC whisker materials that are available in small quantities and the inhalation hazard involved in handling the whiskers (Dunahay, 1993), the glass beads method is generally preferred (Potvin and Zhang, 2010), while the SiC whiskers method is not widely used in either freshwater or marine algal genetic engineering.

3.7. Microinjection

As a direct physical method that is able to penetrate intact cell walls, the microinjection method does not necessarily require a protoplast regeneration system. Additionally, microinjection allows the introduction of DNA (or other substances) under microscopical control into specific targets (Schnorf et al., 1991). Theoretically speaking, the receptor cells of microinjection could be defined as either compartments of a single cell or as defined cells within a multicellular structure, from plants, animals, or microbes. However, due to the difficulties of the immobilization of the algal cells, the complicated manipulation technology, and the low numbers of microinjected cells at a given time that can be achieved (Neuhaus and Spangenberg, 1990), the microinjection method is rarely used in marine algal transformation. A high yield and stable nuclear transformation was achieved in the marine unicellular green alga *Acetabularia mediterranea* by microinjecting SV40 DNA and pSV2neo into the isolated nuclei of algal cells and then implanting the injected nuclei into anucleate cell fragments of the same species (Neuhaus et al., 1986). This established and high yield method was successfully used to tackle the problem of the nuclear transport of algal proteins (Pfeiffer et al., 2009). Regardless of its complicated and delicate procedure, microinjection, could be considered to be a highly efficient and low cost transformation method for marine algae.

3.8. Artificial transposon method

Transposons, which are mobile DNA elements originally discovered in maize, have been strong genetic tools for both prokaryotic and eukaryotic lives (Haapa et al., 1999). Artificial transposons that are extracted and transformed from the essential elements of natural transposons have been broadly developed for in vitro mutagenesis and genetic transformation. It is worth emphasizing that although stable genetic transformation methods have been used successfully in various genera of marine algae, these techniques have the drawback that, in most cases, the genes are integrated randomly into the genome. Such integration may lead to the rearrangement and/or truncation of DNA sequences, thus causing unintentional changes or silencing the expression of the foreign gene. The high frequency of transposition by the artificial transposons method, which can integrate intact foreign DNA into a receptor cell's genome, could partly avoid or compensate for the above-mentioned unintentional changes or silencing of foreign gene expression (Paszkowski and Whitham, 2001, Wu et al., 2011). A modified transformation strategy was applied using a natural Tn5 transposon (Reznikoff, 2008), a transpose, and a cation liposome complex by electroporation to improve the transformation efficiency for *Spirulina platensis* (Kawata et al., 2004), which is one of the most commercially important species of microalgae and can be mass cultured in a seawater medium by domestication.

3.9. Recombinant eukaryotic algal viruses as transformation vectors

The large dsDNA viruses that are known to infect eukaryotic algae show promise as genetic vectors in algal biotechnology. The two main groups of eukaryotic algal viruses are the *Chlorella* system, which displays high levels of infectivity and complete pathogenesis (complete lysis of the unicellular host), and the brown algal virus system by which only specialized reproductive cells of a multi-cellular free-living organism are infected and lysed. The viral effect on the entire marine brown algae, where the genome of the virus is likely integrated, is not

pathogenic. The broad host range including other brown algae permits the wide use of this viral transformation vector in genetic transformation (Henry and Meints, 1994). There is an early report of transformation by the microinjection of the marine unicellular green alga *Acetabularia mediterranea* (Langridge et al., 1985). More extensive and comprehensive fundamental studies on eukaryotic algal viruses are necessary, including its genome, infection mechanism, etc., before this exciting approach can become a reality in the future (Leon and Fernandez, 2007; Van Etten and Meints, 1999).

3.10. Agrobacterium tumefaciens-mediated genetic transformation

A. tumefaciens-mediated genetic transformation genetically transforms plants by transferring and integrating a portion of the resident Ti-plasmid with the large segment DNA up to 150 kb to a plant nuclear genome with the assistance of several virulence (Vir) proteins for T-DNA transfer, nuclear targeting, and integration into the plant genome (Gelvin, 2000; Tzfira and Citovsky, 2006). The first report of a stable genetic transformation by *A. tumefaciens* in algae was conducted in the marine red seaweed *Porphyra yezoensis* (Cheney et al., 2001). The transformation frequency of gene transfer to the nuclear genome of the freshwater green alga *C. reinhardtii* by *A. tumefaciens* was 50-fold higher than that of the glass bead transformation (Kumar et al., 2004). The transformation systems in the marine microalgae *Nannochloropsis* sp. and *Dunaliella bardawil* were also recently established with this method (Anila et al., 2011; Cha et al., 2011). In the *Dunaliella* study, the transformation frequency obtained (41.0 ± 4 cfu per 10^6 cells) was no higher than those reported for glass beads transformation and electroporation, but the transformants obtained were found to be stable for 18 months (Anila et al., 2011). It was found that cinnamic acid, vanillin and coumarin produced higher percentages of GUS positive cells in *Nannochloropsis* sp. transformations compared to acetosyringone, providing possible alternative *Agrobacterium vir* gene inducers that are more potent than the commonly used acetosyringone (Cha et al., 2011).

The reliable *Agrobacterium*-mediated transgenic system for transforming large DNA segments (>100 kb) into marine algae would make it feasible to introduce a natural gene cluster or a set of formerly separate foreign genes into a single locus of the nuclear genome (Hamilton et al., 1996). This strategy could provide a sufficient number of genes for crop protection (crop protection will be discussed in Section 6) when marine algae are in the outdoor mass-culture and constitutes an entirely new metabolic pathway for novel bio-molecule production, metabolic engineering and synthetic biology in algal cells. However, the efficiency of this method is highly dependent on many elements, such as the *Agrobacterium* strain being used, the plasmid vectors, and the extent of virulence (*vir*) gene induction. (Opabode, 2006). This method is also technically challenging because of the large size and low copy number of Ti plasmids, which leads to difficulties in plasmid isolation and manipulation (Meyers et al., 2010). Several developed and advanced binary vector systems are attempting to mitigate these problems and help integrate multiple foreign genes into the same T-DNA (Lee and Gelvin, 2008, Tzfira et al., 2005). The limited applicable genera of marine algae indicate the importance of understanding the molecular mechanisms of *A. tumefaciens*-mediated marine algal (or possibly including freshwater algae) nuclear genome transformation.

4. Vector construction and gene selection strategies

4.1. Vector construction: promoter selection and codon usage

Vector element construction is one of the crucial parts in determining the stability and frequency of exogenous DNA expression in algal expression systems. In marine cyanobacteria, shuttle vectors, which are vectors constructed from a cyanobacterial chromosome segment, and cyanophages are the main possible sources of the vector backbone. In eukaryotic algae, vectors are typically constructed based on their own chromosome segment. Additionally, several vectors from *E. coli* and a few constructed vectors from high plants are sometimes used in marine algal transformations.

Promoter availability and selection is a critical factor in genetic transformation. The CaMV35S and SV40 promoters from viruses are broadly used, particularly when lacking the necessary genetic information of the endogenesis promoters in some marine algae (Anila et al., 2011, Liu et al., 2003, Qin et al., 2004, Wang et al., 2010). Apart from the universal promoters from viruses, the endogenesis promoters from specific marine algae are considered to be the most efficient when constructing a vector. The diatom fucoxanthin-chlorophyll a/c binding protein gene (*fcp*) promoter is effective in marine diatoms and other marine algae (Apt et al., 1996, Li et al., 2009, Miyagawa-Yamaguchi et al., 2011, Qin et al., 2004, Zaslavskaia et al., 2000). The duplicated carbonic anhydrase 1 (*DCY1*) promoter was identified and used for stable nuclear transformation in *D. salina* (Li et al., 2010, Lu et al., 2011). The endogenesis PyAct1 (5′ upstream region of the actin1 gene from *P. yezoensis*) promoter was also confirmed to be effective in the transient gene expression of 12 red seaweed species (Hirata et al., 2011, Takahashi et al., 2010). In *Nannochloropsis* sp. transformation, the endogenous promoters were developed from two unlinked violaxanthin/chlorophyll a-binding protein (*VCP*) genes, VCP1 and VCP2. The VCP1 promoter is a unidirectional promoter, while the VCP2 promoter is bidirectional (Kilian et al., 2011). The endogenous promoter of a nuclear encoded plastid-targeted protein Rubisco SSU (*rbcS*) from the chlorarachniophyte *Lotharella amoebiformis* was isolated and applied to transgenic research (Hirakawa et al., 2008).

Proteins are often difficult to express outside of their original context. They might contain codons that are rarely used in the desired host, originate from organisms that use non-canonical code or contain expression-limiting regulatory elements within their coding sequence (Gustafsson et al., 2004). These problems are typically observed when transferring exogenesis genes to several genomes of marine algae. As is the case for most heterologous genes, optimizing the codon usage of algae-destined transgenes to reflect this bias increases their expression efficiency by increasing their translation rates and may decrease their susceptibility to silencing (Heitzer et al., 2007, Potvin and Zhang, 2010). In prokaryotic or prokaryotic-derived genomes, such as chloroplasts from eukaryotic algae, codon bias is one of the most critical elements for protein expression (Surzycki et al., 2009). The importance of codon optimization in marine algal genetic transformation applications is increasingly acknowledged in recent transgenic research (Lerche and Hallmann, 2009, Takahashi et al., 2010). Several free software and web applications have recently been developed to estimate and optimize the codon usage of sequences (Potvin and Zhang, 2010). Today, the rapidly developing field of synthetic biology is endowing codon optimization as a necessity, and it is becoming increasingly significant for *de novo* DNA synthesis with other gene design principles (McArthur and Fong, 2010, Welch et al., 2009).

4.2. Reporter and marker genes

The protein expressed by a reporter gene is sensitive, intuitionistic and easy to detect; thus, it can be used to demarcate the transformation frequency, the expression efficiency and the stability of the transformed foreign gene, as well as to determine the protein locality in the transformed cells. The widely used reporter genes in marine algal transformation are *GUS* and *lacZ*. The *GUS* gene encoding the β-glucuronidase is typically selected as an effective reporter for transient and stable expression in marine algae, e.g., *D. salina* (Tan et al., 2005), *Amphidinium* sp., *Symbiodinium microadriaticum* (ten Lohuis and Miller, 1998), *T. weissflogii* (Falciatore et al., 1999), *Ectocarpus* sp. (Cheney and Kurtzman, 1992), *Porphyra yezoesis* (Hirata et al., 2011, Kuang et al., 1998, Liu et al., 2003), *Ulva lactuca* (Huang et al., 1996), *L. japonica*

(Li et al., 2009, Qin et al., 1994), *Undaria pinnatifida* (Qin et al., 1994). However, because the substrate of this gene has an impermeable membrane, it is toxic to the transformed cell and damages the cellular ultrastructure during the dyeing process. In addition, in higher plants and marine seaweeds, it has been detected that there was a weak background of the *GUS* gene and, thus the negative control must be set to eliminate the background signal. The *LacZ* gene exists in several bacteria and is similar to the *GUS* gene because it is more effective in conducting anti-bacteria processes and requires a negative and blank control to eliminate the background. The *lacZ* essay is applied in many marine algae, such as red alga *P. haitanensis* (Zhang et al., 2010) and brown alga *L. japonica* (Jiang et al., 2003, Qin et al., 1998). The *Luc* gene encoding *luciferase* is another reporter gene usually applied in a freshwater microalgae and the marine diatom *P. tricornutum* (Falciatore et al., 1999). The green fluorescent protein (*GFP*) of the jellyfish *Aequorea victoria* has been used as a universal reporter of gene expression and in subcellular localization analyses in various marine algae (Hirakawa et al., 2008, Miyagawa-Yamaguchi et al., 2011, Poulsen et al., 2006, Takahashi et al., 2010, Wang et al., 2010, Watanabe et al., 2011, Zaslavskaia et al., 2000). However, because algae typically have endogenous photosynthetic pigments and other fluorescent substances, the expression of *GFP* requires a strong promoter to prevent the interference from the interior fluorescence background.

Transformation protocols require effective selection markers to discriminate successful transformants from transformed cells. The majority of the selectable markers contain two types: one type includes genes with a resistance to antibiotics. These selection genes, conferring antibiotics of high plants or anti-herbicides, are most commonly used for the selection of marine algal transformants. In contrast to high plants, marine algae are not sensitive to neomycin and kanamycin but are typically sensitive to chloromycetin, hygromycin, and herbicide glufosinate. The other type of marker is the homologous complementation of metabolic mutants. This method may be particularly useful for chloroplast transformations. Although lists of selectable markers in microalgae have been compiled in past reviews (Griesbeck et al., 2006, León-Bañares et al., 2004, Walker et al., 2005), novel markers have since been developed, such as Phytoene desaturase (*PDS*) (Steinbrenner and Sandmann, 2006) and ARG9 genes (Remacle et al., 2009). There are concerns regarding the biosafety of antibiotic- and herbicide-resistant genes when releasing the transgenic plants to the environment, and efforts are being made to develop alternative marker systems and standardize marker-free selection systems (Manimaran et al., 2011). The selection marker removal may significantly reduce the public acceptance of genetically modified plants (Miki and McHugh, 2004). Several marker elimination methods in higher plants have been developed during these years including co-transformation, which is usually applied in *Agrobacterium*-mediated transformation (Sripriya et al., 2008) and various site-specific recombination methods that eliminate the selection marker by deleting or inverting the marker gene with the help of an enzyme recombinase (Cotsaftis et al., 2002; Darbani et al., 2007, Kopertekh et al., 2004, Ow, 2002). The marine algal genetic transformation will certainly meet these biosafety issues (more discussion can be found in Section 6) when cultivating transgenic microalgae in an open pond of a coastal area or transgenic macroalgal sporophytes in the open sea. Because of the differences in the genetic background, growth and development mode, propagation and breeding type between high plants and marine algae, as well as the particularity of marine environments, the marker elimination methodology applied in marine algal genetic transformation needs to be considered synthetically, and some changes need to be made based on the changes applied in high plants.

4.3. Gene copy number and homology-dependent gene silencing

The expression levels of transgenic genes in marine algae are inconsistent and difficult to predict. The significant reasons for unpredictable variation arise from inconsistencies in the number of integrated transgene copies and the subsequent homology-dependent gene silencing. Single-copy transformants are preferable and desirable because of their higher expression level and they are much more predictable, while, to some extent, this expectation is ideal in marine algal transformation. Silencing, which increases with a growing number of integrated gene copies, occurs at the transcriptional or post-transcriptional level and is believed to have originated as a defense mechanism of plants against viruses and as a means of regulating gene expression (Baulcombe, 2004; Depicker and VanMontagu, 1997; Marenkova and Deineko, 2010; Potvin and Zhang, 2010). Electroporation commonly results in highly variable integrated transgene copy numbers and low-copy transformants. *Agrobacterium*-mediated transformation typically leads to low copy numbers and higher single-copy transformants, as discussed previously in Section 3. 10. The direct DNA-transfer methods such as glass beads and biolistic bombardment usually lead to a large number of integrated gene copies in the receptor algal genome, which may increase silencing effects. Single or low number copy transgenic lines can be obtained by transformation cassettes and control the amount of cassette DNA when using the biolistic method (Lowe et al., 2009, Yao et al., 2006).

4.4. RNA interference technology

Gene silencing can occur either through repression of transcription, termed transcriptional gene silencing, or through mRNA degradation, termed post-transcriptional gene silencing (Angaji et al., 2010). Because of its high specificity and efficiency, RNA interference has been proven to be an invaluable tool for analyzing the biological function of the target gene and adjusting the metabolism process considerably by sequence-specific knockdown (Cerutti et al., 2011; Schramke and Allshire, 2005, Waterhouse et al., 1998). A wide range of core RNAi machinery components, which promotes the transient gene silencing and stable gene repression experiments, was identified in the marine algae, red alga *P. yezoensis* (Liang et al., 2010), green alga *D. salina* (Jia et al., 2009), diatom *P. tricornutum* (De Riso et al., 2009), *T. pseudonana* (Armbrust et al., 2004), and brown alga *Ectocarpus siliculosus* (Cock et al., 2010). To investigate the potential of double-stranded RNA interference with gene expression in *D. salina*, a plasmid was constructed to express hairpin RNA containing sequences homologous to phytoene desaturase genes that were transformed into *D. salina* by electroporation for the transient suppression of gene expression (Sun et al., 2008). RNA interference was applied in *P. tricornutum* to ensure that the genes were related to the uridine-5′-monophosphate synthase process and revealed the potential usage of this gene silencing and complementation system as a powerful genetic tool for this marine alga (Sakaguchi et al., 2011).

5. Current progress of photobioreactor technologies for marine algae

5.1. Marine algal photobioreactor: co-development of the ‘open’ and the ‘closed’

Marine algae is presently the best choice for cell factories of recombinant protein productions and the source of future biofuel because they have simple and inexpensive growth requirements (free seawater, inexpensive nitrogen, phosphorus, and carbon sources), rapid growth rates with sufficient light, and can be used in the marginal land of coastal area or can even be used in the sea. The key components to promote the algal growth in photobioreactors are nutrients, light, turbulence, and contamination prevention. The high density and large-scale cultivation for marine algae is the only path for the development and application of these bioresources. The photobioreactor design is a key element for this process. To some extent, the development of transgenic marine

algae (or even algal biotechnology) is extremely dependent on the R&D of the photobioreactor.

Applied phycologists have generally distinguished between open ponds and closed photobioreactors, where the latter implies that light does not impinge directly on the culture's surface and that there is no direct contact between the culture and the atmosphere (Tredici, 2007) for growing microalgae and the micro-generation or cells of macroalgae (Rorrer and Cheney, 2004). Open ponds, commonly used for ultra thin-layered cascade systems, are extensively used in algal biotechnology applications, including the raceways, sloping and cascade systems (Grobbelaar et al., 1995). However, closed photobioreactors have several advantages over medium light-path open ponds, including higher light utilization efficiencies, nutrient uptake, volumetric biomass concentrations, lower compensation light/dark ratios or respiratory losses, less contamination and competition with alien algae and less water losses during the culture system (Grobbelaar, 2009).

5.2. Photoioreactor for marine microalgae

A 200-liter unit flat glass reactor was designed to optimize the outdoor mass production of *Nannochloropsis* sp. The highest areal productivity was obtained in a 10 cm light-path reactor. The cost-effectiveness of this photobioreactor was compared carefully with the open pond (raceway) for factors including volume, ground area, and harvested cell density. The optimal population density in the 10 cm plate reactor was obtained with a daily harvest of 10% of the culture volume yielding an annual average of ca. 12.1 g dry wt. m^{-2} day^{-1} or 0.24 g m^{-3} day^{-1} (Richmond and Cheng-Wu, 2001). The performance of the *D. salina* cultures outdoors in a closed tubular photobioreactor was assessed. The maximal biomass productivity (over 2 g dry wt. m^{-2} day^{-1} or 80 g^{-3} day^{-1}) was achieved (Garcia-Gonzalez et al., 2005). This productivity value is higher than what the same research group carried out in an open pond system demonstrating that the closed tubular photobioreactor has significant advantages over the open ponds in the marine algal yield rate (Garcia-Gonzalez et al., 2003). The efficiency and reliability of closed photobioreactors for culturing coccolithophorid algae were also evaluated in open raceway ponds and several types of closed photobioreactors (Moheimani and Borowitzka, 2006, Moheimani et al., 2011). Although the yield rate or biomass concentration at harvesting is typically high when using a closed photobioreactor, the very low cost for construction and maintenance and the fewer overheating problems experienced and super dissolved oxygen concentrations make the open pond system an attractive option when considering the cost control and economic income for large-scale marine microalgal culture.

5.3. Photobioreactor for marine macroalgae

Photobioreactor design is also one of the key elements and can be a barrier to bioprocess technology for marine macroalgae (Rorrer and Cheney, 2004, Rorrer et al., 1998). Gametophytic expression systems for transgenic *L. japonica* and *U. pinnatifida* were established using bubble-column and airlift closed photobioreactors. Two functional genes (human acidic fibroblast growth factor gene and tachyplesin gene from *Tachypleus tridentatus*) were successfully integrated into algal nuclear genomes and expressed in this system (Deng et al., 2008, 2009). Although the expression efficiency of tachyplesin in *L. japonica* gametophtes is lower than the expression efficiency in *E. coli* and *Bacillaceae* expression systems, the expressed recombinant proteins possess their natural biological structure without a renaturation process. This trait gives gametophytic expression systems a remarkable advantage in the cost reduction in the post-translational medication process, which constitutes a large portion of the cost for transgenic downstream bioprocesses.

6. Perspectives

6.1. Key elements for evaluating marine algal expression systems

The crucial and fundamental issue of marine algal biotechnology is the 'seed' problem. The ultimate aim of marine algal genetic transformation is to provide future industrialized large-scale cultivation with seeds that display powerful and attractive traits to produce valuable products for algal economic feasibility and remove CO_2, heavy metal as bioremediation for their ecological contribution. Several genera of marine algae have been successfully cultivated for commercial exploitations, such as *Dunaliella, Porphyridium*, *Nannochloropsis, Laminaria*, *Undaria*, *Porphyra* and *Gracilaria*. To establish an effective expression system for marine algae, several factors need to be taken into account: ① economic target, which includes the cost and profit calculations; ② technological methods, including the vector's construction and selection, selection of transformants; ③ engineering design, which includes the bioreactor's design, running and maintenance; ④ the safety issues of transgenic marine algae (Lin et al., 2011).

6.2. Crop protection

Like terrestrial monocultures, large marine algal monocultures will be invaded by pests and pathogens; thus, crop protection is a major challenge in large-scale algal cultivation sustainability (Hannon et al., 2010), which will be increasingly significant with the development and scale-up of marine algal cultivation. The open photobioreactors will have more significant challenges regarding contamination than the closed photobioreactors, which have the potential to minimize contamination due to their nearly axenic filtrate system for water and air. However, this comes at a high capital expense. There are alternative solutions for crop protection. Antimicrobial peptides, which are expressed from only a single heterologous gene, provide a solution for a broad-spectrum ranging from anti-bacteria, -fungi and -protozoan (Farrokhi et al., 2008; Rahnamaeian and Vilcinskas, 2012). These peptides have been successfully integrated and transformed into the nuclei and chloroplasts of higher plants (DeGray et al., 2001, Jan et al., 2010, Lee et al., 2011). To prevent the prey from invertebrates, several allelochemicals from cyanobacteria can be introduced and expressed to defend against potential predators and grazers (Berry et al., 2008; Rastogi and Sinha, 2009). An ecological method could also be considered according to the relationship in the ecosystem (Hansen et al., 1993, Koski et al., 2005).

6.3. Biosafety assessment

The issue of the biosafety of transgenic marine algae contains two components: one component is to eliminate harm to human health, and the other component is to be environmentally friendly. For the human health concerns, the biosafety evaluation could refer to the methods applied in higher plants (Kikuchi et al., 2008, Maliga and Maliga, 2006, Oh et al., 2011, Ramessar et al., 2007) to guarantee that there are no poisonous substances and allergens. However, with the aim to remove the potential threats to human health from the genetically transformed marine algae, it is necessary to develop 'completely marine algae-derived' vectors, which includes searching for endogenous promoters, establishing a directional foreign gene integration platform according to algal homologous recombination, substituting antibiotic selection genes for an endogenous selection system of reverse mutation, and replacing animal virus DNA sequences with algal endogenous vectors or algal virus DNA sequences. In addition, it is worth paying greater attention to the issue in which the transformed gene escapes from the marine algal transformation and the expression system because the cultivation environment of marine algae is an open and flowing body of seawater. It is critical to assess the transgenic algae crossbreed with the domestic species/strains by a set of comprehensive

observations and detection systems on the status and changes of the domestic algal genetic background. *L. japonica* is the only species of seaweed that has been carried out in the biosafety assessment. *L. japonica*, a species endemic to the Sea of Japan and imported to the northern part of China in the 1920s (Tseng, 2001), has less of an effect on the domestic relative species. Thus, the biosafety evaluation on transgenic male and female sporophytes was conducted with safe containers on the sea and harvested before maturation of the sporangia (Qin et al., 2005).

7. Conclusions

Synthetic biological devices have great potential in many biotechnological applications. However, the functional complexity of synthetic devices has been limited by the available design tools (Muers, 2012; Purnick and Weiss, 2009). The prospect of being able to design and build novel algal biomolecules and components, metabolic networks, and even to rewire and reprogram photoautotrophs is extremely exciting. The pilot study was conducted by using the model freshwater cyanobacteria *Synechococcus elongates* for designing two-species synthetic biological systems by injection into specific animal cells (Agapakis et al., 2011). Cyanobacteria and eukaryotic algae exhibit potential in their abundant material pool and programmable photoautotroph cells for the aforementioned design and construction. It is now essential to develop effective strategies for assembling various and complex devices and modules in marine algal cells into intricate, customizable larger scale systems, such as an artificial and recombinant photosynthetic apparatus to develop innovative approaches to a broader range of applications in the genetic engineering of marine algae for ocean farming and human health, as well as for worldwide energy shortage and global environmental deterioration.

Acknowledgements

The authors thank SOA Public Science and Technology Research Funds Projects of Ocean (Grant No. 20120527 and 200905021-3), NSFC general program (Grant No. 41176144), CAS/SAFEA International Partnership Program for Creative Research Teams 'Typical Process in Coastal Zone Area and Its Effects on Bio-resources' (Grant No. KZCX2-YW-T14), '973' program of China (Grant No. 2011CB200902), '863' Program of China (Grant No. 2009AA10Z106), Promotive Research Fund for Excellent Young and Middle-aged Scientists of Shandong Province (Grant No. 2010BSB02009), and NSF of Shandong Province (Grant No. JQ200914 and 2009ZRB02542), who provided the financial support over the course of this work.

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