CHAPTER 2

LITERATURE REVIEW

2.1 Algae

Algae are unique lower plants with specific characteristics. They are mostly aquatic species with the ability to convert inorganic compounds into organic matter by means of light energy through the process of photosynthesis like other plants but they are much smaller and less structurally complex than land plants (Graham and Wilcox, 2000). According to Graham and Wilcox (2000), a great deal of variation existed in the morphology of algal thallus (algal body) and the most commonly encountered forms were unicells and colonies, filaments, coenocytic or siphonaceous, and parenchymatous and pseudoparenchymatous algae.

Early taxonomists classified all known species into two kingdoms which are plants and animals. At that time, eukaryotic unicellular organisms with chloroplasts were also considered plants (Campbell *et al*., 2008). In 1969, an American ecologist Robert Whittaker argued effectively for a five kingdom system which are Monera, Protista, Plantae, Fungi and Animalia. It places prokaryotes in the Kingdom Monera and organism of the other four kingdoms all consists of eukaryotic cells. The kingdoms Plantae, Fungi and Animalia consist of multicellular eukaryotes that differ in structure, development and models of nutrition (Campbell *et al*., 2004). However, shortly after the widespread adoption of the five kingdom approach, phylogenies based on genetic data began to reveal a fundamental problem with this system. Therefore, the three domain system which consisted Bacteria, Archaea and Eukarya, was adopted. In this system, the green algae (Chlorophyta), red algae (Rhodophyta), brown algae (Phaeophyta), Golden Algae (Chrysophyta), Diatoms (Dinoflagellates), and Euglena

(Euglenoids) were placed in Domain Eukarya. The blue green algae (Cyanophyta) is placed under Bacteria (Campbell *et al*., 2008).

Algae reproduce by a variety of means, both sexual and asexual. In sexual reproduction, plasmogamy which is the fusion of haploid reproductive cells (gametes), is followed by karyogamy (nuclear fusion), to form a diploid zygote. In asexual reproduction, a number of common processes and structures are involved. Among them are cellular bisection, zoospore and aplanospore formation, autospore or monospore production, autocolony formation, fragmentation and akinetes (Graham and Wilcox, 2000).

The different groups of algae contain combinations of pigments belonging to chlorophylls, carotenoids and the phycobiliproteins (Bold and Wyne, 1978). The pigmentation of higher plants is very similar to that of the Chlorophyta, the phylum from which land plants are thought to have evolved. The dominant pigments are chlorophyll-a, colouring the Chlorophyta green; carotene and diatomin giving the characteristic yellowish-green or brown colour of the Chrysophyta; fucoxanthin, which colours the Phaeophyta yellow-brown; phycoerythrin, which colours the Rhodophyta red; and phycocyanin and chlorophyll, which together colours the Cyanophyta blue green (Lewin, 1962). The final products of photosynthesis also differs for each group; the Chlorophyta produces starch, the Phaeophyta forms laminarin, while Cyanophyta produces cynophycean starch, and the Rhodophyta forms rhodophycean starch (Bold and Wyne, 1978).

2.2 *Chlorella* species

The simple and common green microalgae of the genus *Chlorella* are placed within the order Chlorococcales and family Chlorellacea (Graham and Wilcox, 2000). The genus of *Chlorella* includes species like *Chlorella vulgaris, Chlorella pyrenoidosa, Chlorella*

sorokiniana, Chlorella ellipsoidea, and Chlorella regulararis. *Chlorella* was believed to be a form of life that emerged over two billion years ago after the photosynthetic bacteria. Moreover, fossils from the Pre-Cambrian period clearly indicated the presence of *Chlorella* (Balch and Balch, 1990).

Wardburg (1919) discovered that pure cultures of these fast growing micro-organisms could be used as ideal experimental materials, for research on photosynthesis, nitrate reduction, physiology and biochemistry. Some *Chlorella* species grow symbiotically in various organisms such as *Hydra, Paramecium* and some other protozoa and lichens (Graham and Wilcox, 2000). The length of the *Chlorella*'s life cycle depends upon the strength of sun light, temperature and nutrients (Balch and Balch, 1990).

In the *Chlorella* cell, the nucleus, starch grains, belt-shaped chloroplast where photosynthesis takes place and the mitochondria that generate energy for cell survival are present (Balch and Balch, 1990). According to Graham and Wilcox (2000), the chloroplast is parietal and there is a pyrenoid in some but not all species.

The asexual reproduction of *Chlorella* is by the formation of four autospores and the sexual production is not known (Graham and Wilcox, 2000). Since *Chlorella* is asexually reproduced therefore there is no clear distinction between male and female cells (Balch and Balch, 1990). The daughter cell grows to become a mother cell and divides to produce four daughter cells (Graham and Wilcox, 2000). Since, *Chlorella* undergo a simple cell division cycle, it enables them to complete their life cycle within a few hours. This also allows much more rapid development and demonstration of production processes than with other agricultural crops (El-Sheekh, 1999).

The surface of some *Chlorella* is covered with cell walls, which comprises mainly cellulose, glucose and mannose whereas other species possess glucosamine walls (Takeda, 1991). *Chlorella* cell walls are rigid with low digestibility and are rich with all eight essential amino acids, vitamins B12, beta-carotene, calcium, iron and chlorophyll (Pickett-Heaps, 1975).

2.3 Importance of *Chlorella* species

2.3.1 Nutrient Composition

Chlorella contains high vitamin contents including Vitamin B complex such as B1, B2, B3 and folic acid. This potential microalga is available in powdered tablets and capsules for health consumption (Balch and Balch, 1990). *Chlorella* also contains high amount of lipids and amino acids (Satin, 2004). The lipids of *Chlorella* consist of high content of docosahaxaenoic (DHA) and eicosapentaeneoic (EPA) acids which are essential polyunsaturated fatty acids (PUFA) in the diet of children for brain development. These PUFA aids in reducing the risk of coronary heart disease and alleviate inflammatory disease (Balch and Balch, 1990).

2.3.2 Bio-remedy

Chlorella has been used as energy-producing food in Asia for many centuries. In Japan, it is traditionally used as a treatment for hypoglycaemia, asthma and constipation. *Chlorella* is also used as adjunct supplement during radiation treatment, and it is also known to protect the body from ultraviolet radiation. This microalga has been touted as an effective therapy for elevated and adjunct treatment for cancer because anti-tumor properties in fighting breast cancer were found in *Chlorella* (Tanaka, 1998). Phang and Chu (2004)

reported that *C. vulgaris* can be used to treat rubber and palm oil effluents in high rate algal pond. Besides that, *C. vulgaris* can also be used to treat waste water because it is tolerant to several heavy metals (Phang and Chu, 2004).

2.3.3 Economic Benefits

Chlorella can be cultured inexpensively on a large scale because it requires only a limited amount of minerals and sunlight. Some species grow relatively fast, dividing up to two to nine times per day depending on the light intensity and temperature (Kim *et al*., 2002). Hawkins and Nakamura (1999) also mentioned that *Chlorella* could be easily grown inexpensively in defined medium of simple salts with KNO_3 or ammonium chloride as a nitrogen source and atmospheric CO_2 as a carbon source. *Chlorella* can be grown to achieve 1.5 to 2.5 g/day/L if grown in shallow tanks (Hawkins and Nakamura, 1999). Walker *et al*. (2005) also reported that *Chlorella* cultures can achieve high cell densities under conditions of high light and aeration, and can be grown to volumes of several megalitres. Dawson *et al*. (1997) reported that these are the characteristics that make *Chlorella* potentially useful as a bioreactor for synthesizing compounds of pharmaceutical interest as well as, for basic research such as, enzymes, hormones, viral vaccines, monoclonal antibodies, immunoregulators, growth, blood clotting factors and products like Taxol, an anticancer agent. *Chlorella* and several other microalgae like *Dunaliella, Spirulina*, and *Haematococcus* were also cultured for the extraction of valuable compounds like carotenoids which is highly demanded product in the market (Borowitzka, 1995).

2.3.4 Importance in Molecular Research

Escherichia coli is the most widely used heterologous expression system, but it has several limitations, such as poor or no expression of some proteins, proteins that lack

biological activity, proteins that are toxic to *E. coli*, and formation of insoluble inclusion bodies (Friehs and Reardon, 1993). Similar problems have also been observed in yeast expression system. It has been reported that *Chlorella* could be used as a good system for foreign protein expression. This is because *Chlorella* is a eukaryote, which therefore can synthesize complex protein that requires post-translational modification, in order to become biologically active (Kim *et al.*, 2002). According to Hawkins and Nakamura (1999), eukaryotic algal species like *Chlorella* have not been extensively utilized for the purposes of protein expression, although they do have characteristics that would be desirable as a potential system for high level expression of protein.

Chlorella offers an alternative system to study the molecular biology of many important cellular processes for lower eukaryotes as well as higher plants. The characteristics that make *Chlorella* as a useful model are similar metabolic pathways to higher plants; the cells can be synchronized; easy to manipulate the cell's environment; and cell cultures are easy, rapid and inexpensive to grow (Dawson *et al*., 1997).

2.4 Genetic Engineering and Manipulation of Algae

According to Walker *et al*. (2005), the last few years witnessed significant advances in the field of algal genomics. The availability of the genome data of algae may serve as a powerful catalyst for the development and application of recombination technology in the field of phycology. The complete DNA sequences of algae provide a rich source of DNA elements such as promoters that can be used for transgene expression as well as an inventory of genes that are possible targets for genetic engineering projects aimed at manipulating algal metabolism. It is therefore not surprising that significant progress in the genetic engineering of eukaryotic algae is being achieved (Walker *et al*., 2005).

At present, the algal industry is worth several billion US dollars world wide and the challenges facing the industry include a stable supply of high quality raw materials, new algal product development and continuous search for new algal species with novel properties. Large-scale cultivation of economically important algal species and genetic engineering of desirable algal strains are approaches to ensure steady supply of quality raw material to the algal industry (Ask, 2003; Minocha, 2003; Qin *et al*., 2004). There has been interest in the mining of algal species for novel pharmaceuticals, and in the near future, transgenic microalgae may be exploited as cell factories for the production of valuable recombinant products such as vaccines, specialty oils and novel carotenoids (Moore, 1999; Skulberg, 2000; Leon-Banares *et al*., 2004).

Difficulties in genetic engineering of algae were recognized in the early 1990's (Saga, 1991). There was a poor knowledge on how to introduce foreign DNA into algal cells as well as on how to regenerate and select transformed plants. In China, genetically transformed economic algae such as *Laminaria*, *Undaria*, *Porphyra*, *Gracilaria*, *Ceramium*, and *Grateloupia* have been produced since 1991, and visible progress has been made in the world towards establishing a transformation model for research (Qin *et al*., 2004). *Porphyra yezoensis* or nori has been the interest of genetic engineering not only in China but in Japan and the USA (Cheney *et al*., 2004). There have been also reports of the transient transformation of genetically transformed green alga, *Ulva lactuca* (Huang *et al*., 1996). In 2005, Gan reported a transient transformation system of the red alga, *Gracilaria changii* with the *lacZ* gene using particle bombardment and *Agrobacterium tumefacien*-mediated transformation which has been filed for patents.

According to Walker *et al*. (2005), fundamental and applied research are crucial for the development of molecular genetic techniques for each algal species, in order to allow the *in vivo* analysis of gene function and regulation, manipulation of endogenous genes, and the

introduction and expression of foreign genes. Over the last ten to 15 years, sophisticated molecular toolkits have been developed for several laboratory models, such as the green algae *Chlamydomonas reinhardtii, Chlorella, Dunaliella salina, D. tertiolecta* and *Volvox carteri* and the diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Walker *et al*., 2005). This has resulted in significant advances in the understanding of basic biology in these organisms (Kirk, 1998; Harris, 2001; Falciatore and Bowler, 2002). Since then, DNA transformation of the green microalgae *D. salina* and *Haemotococcus pluvialis* provided most of the world's supply of natural β-carotene and astaxanthin, respectively (Curtain, 2000; Guerin *et al.*, 2003). The feasibility of producing high-value recombinant proteins in several *Chlorella* species and *C. reinhardtii* demonstrates that there is a great possibility of using transgenic algae as vectors to deliver either vaccines or toxins to animals that feed on algae (Walker *et al*., 2005). In addition, microalgae can be used as model eukaryotic hosts, especially in cases where specific genes cannot be expressed in yeast. Furthermore, microalgae can be engineered to express high quality mammalian proteins such as hormones or antibodies. Xue *et al*. (2001) reported that *D. salina* could express the tumor necrosis factor (TNF). Thus, the development of a method to deliver the TNF gene into *D. salina* could lead to a medical breakthrough because the TNF gene is involved in infectious and immuno-inflamatory diseases (Louis *et al*., 1998).

Transgenic algae are suitable for phototrophic and heterotrophic conditions (Leon-Banares *et al*., 2004). Culturing of algae in illuminated bioreactors is expensive and inefficient; however, a technique for converting an obligate photoautotroph to a strain capable of heterotrophic growth on glucose through the introduction of a human glucose transporter gene has been demonstrated for the diatom, *Phaeodactylum tricornutum* (Zaslavskaia *et al*., 2001). This trophic conversion of a microalga would improve the efficiency and cost of biomass production by allowing heterotrophic culture in conventional fermenter rather than photoautotrophic growth in photo-bioreactors or in open pond systems,

where the supply of light is essential compared to a heterotrophic culture which can grow even 24 hours without the necessity of light (Walker *et al*., 2005).

The increasing importance of genetic transformation techniques in algal biotechnology can be seen by the recent filing of patents relating to the genetic manipulation of algae and the establishment of several biotechnology companies (Dunahay *et al*., 1997; Allnutt *et al*., 2000; Cheney, 2000; Sayre *et al*., 2003; Xue *et al*., 2001). Moreover, funding bodies are recognizing the importance of algal molecular research and are actively supporting a number of algal genome initiatives. This support is catalyzing efforts to develop and apply recombinant techniques to a wide range of algal species. It is therefore timely to look into the progress and future possibilities of algal transgenics (Walker *et al*., 2005).

2.4.1 Genetic Transformation of *Chlorella*

Chlorella has been successfully transformed with many types of genes like the firefly luciferase, β-glucoronidase, nitrate reductase, human and flounder growth hormone, hygromycin and geneticin resistance genes (Table 2.1). The first genetic transformation of *Chlorella* was reported by Jarvis and Brown (1991), when they reported transient expression of the firefly luciferase in the protoplasts of *Chlorella ellipsoidea.* In 1994, Miller *et al*. and Maruyama *et al*. transformed *C. sorokiniana* and *C. saccharophila*, respectively, using electroporation technique. Then, Dawson *et al*. (1997) reported on the first stable transfection of *Chlorella* by using the nitrate reductase gene from *C. vulgaris* to rescue the nitrate reductase-deficient *C. sorokiniana* mutants. Chow and Tung (1999) transformed *C. vulgaris* cells by electroporation using the hygromycin (*hpt*) resistance gene. In the same year, El-Sheekh (1999) was able to transform *C. kessleri* with the β-glucoronidase (*GUS*) gene.

The neutrophil peptide 1 (NP-1) from rabbit neutrophils has the broadest antimicrobial and cytotoxic spectrum. It could also be a good candidate to be developed as a novel antimicrobial agent for medical and agricultural purposes (Chen *et al*., 2001). In 2001, Chen *et al*. developed a highly efficient expression of rabbit neutrophil peptide-1 gene in *C. ellipsoidea* cell. The expression of the NP-1 gene in *C. ellipsoidea* exhibited significant inhibition towards gram-positive bacteria, gram-negative bacteria and fungi (Chen *et al*., 2001).

The advancement of genetic transformation especially for *Chlorella* species could help to reduce the quantity of heavy metals existing released by industries into the aquatic ecosystem. Huang *et al*. (2006) transformed *Chlorella* with the mercury reductase (MerA) gene via PEG and electroporation. The integration and expression of the MerA gene in *Chlorella* was a new approach for mercury phytoremediation, which can be used as in the treatment of waste water (Huang *et al*., 2006). Besides that, *Chlorella* can also be transformed to express pesticidal polypeptides. According to Borovsky (2003), mosquito is the cause of many diseases like malaria, dengue and yellow fever. Trypsin-modulating oostatic factor (TMOF) is a mosquito decapeptide that terminates trypsin biosynthesis in the mosquito gut (Borovsky, 2003). Therefore, when *Chorella* was transformed with the GFP-TMOF gene via PEG and fed to *Aedes aegypti* 2nd instar of the larvae, the transformed *Chlorella* caused 95% mortality larvae within 72 hours, suggesting it was effective as a mosquito larvicide (Borovsky, 2003).

Viral diseases like Yellow head virus (YHV), Taura syndrome virus (TSV), Infectious hypodermal and hematopoietic necrosis virus (HIHNV), White spot syndrome virus (WSSV) and White spot bacilloform virus (WSBV) cause large economic losses in crustacean aquaculture (Dhar and Allnutt, 2004). Allnutt *et al*. (2000) reported that shrimps developed resistance towards the WSSV and TSV viruses when it was fed with *Chlorella vulgaris*

strains that were transformed with the WSSV gene and TSV gene. The development of therapeutics to prevent these viral diseases from infecting shrimps could reduce pandemics that have affected global penaeid shrimp farming (Dhar and Allnutt, 2004).

In 2002, Kim *et al*. was able to achieve stable integration and functional expression of the flounder growth hormone (fGH) in the transformed microalga, *Chlorella ellipsoidea*. It was reported that the growth of the flounder fry increased 25% within 30 days after being fed with *C. ellipsoidea* that was transformed with the fGH gene (Kim *et al*., 2002). In 1999, Hawkins and Nakamura also tried a similar approach to introduce the human growth hormone (hGH) into *Chlorella* but the expression was not stable and the biological activity was not tested.

Table 2.1 Genetic transformation of several *Chlorella* spp. from 1991 to 2006.

Host Species	Genes	Methods	References
C. ellipsoidea	firefly luciferase	PEG	Jarvis and Brown, 1991
C. saccharophila	*GUS*	electroporation	Maruyama *et al*., 1994
C. sorokiniana	*GUS*	electroporation	Miller *et al*., 1994
C. sorokiniana	nitrate reductase	Biolistics	Dawson *et al*., 1997
C. vulgaris and *C. sorokiniana*	hGH	PEG and electroporation	Hawkins and Nakamura, 1999
C. vulgaris	*hpt*	electroporation	Chow and Tung, 1999
C. kessleri	*GUS*	Biolistics	El-Sheekh, 1999
Chlorella sp.	GFP-TMOF	PEG	Borovsky, 2003
C. vulgaris	WSSV and TSV	Biolistics	Allnutt *et al*., 2000
C. ellipsoidea	NP-1	electroporation	Chen *et al*., 2001
C. ellipsoidea	fGH	PEG	Kim *et al*., 2002
Chlorella sp.	MerA	PEG and electroporation	Huang *et al*., 2006

2.5 Methods of DNA Transfer

2.5.1 *Agrobacterium*-mediated Transformation

Agrobacterium tumefaciens has played a major role in the development of plant genetic engineering and basic research in molecular biology. It accounts for about 80% transgenic plants produced thus far (Wang and Fang, 1998). Initially, it was believed that only dicotyledons, gymnosperm and a few monocotyledons could be transformed by this bacterium, but recent achievements reviewed that many recalcitrant species which are not included in its natural host range such as monocotyledons, fungi, fern, moss and algae could now be transformed (Chan *et al*., 1993; Bundock *et al.,* 1995; Wei *et al*., 2000). However *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods such as particle bombardment and electroporation. It produces less copy number of the transgene which may lead to fewer problems with transgene cosuppression and instability (Koncz *et al*., 1994; Hansen *et al.*, 1997; Wei *et al*., 2000).

2.5.2 Particle Bombardment

Direct biolistic is also known as particle bombardment or biological ballistics. The biolistics is one was by which biological molecules, such as DNA and RNA, are accelerated usually on microcarriers termed microprojectiles, by gun powder, compressed gas or other means. The biological molecules are thus driven at high velocity into the target (Galun and Breiman, 1998). This technique involves the coating of the micron-sized metal particles with desired DNA and accelerated into the cell at high velocities sufficient to penetrate the cell wall but without causing lethal damage so that the desired DNA can be transported into the cell's interior where it becomes detached from the microprojectile and integrated into the cell genome (Taylor and Fauquet, 2002).

Initial reports were restricted to transient expression of marker genes in onion, corn, soybean, wheat and rice but were soon followed by transgenic soy bean and maize plants (Klein *et al*., 1992; McCabe *et al*., 1988; Fromm *et al*., 1990; Gordon-Kamm *et al*., 1990). This technology has also been adapted to transfer exogenous DNA to bacteria, fungi, algae, insects and mammals (Klein *et al*., 1992; Smith *et al*., 1992; Armaleo *et al*., 1990; Mayfield and Kindle, 1990; Cheng *et al*., 1993, Luo and Saltzman, 2000). Luthra *et al*. (1997) documented 200 scientific papers published between 1987 to 1995, which reported the use of particle bombardment technologies in plant science. Taylor and Fauquet (2002) reported that since 1995, more than 250 additional papers have been published in this area, illustrating the continued importance of particle bombardment technology. Bateman and Purton (2000) reported that transformation involving microparticle bombardment has been successfully applied to many unicellular microalga like *Chlamydomonas reinhardtii, Chlorella sorokiniana, C. kessleri, C. vulgaris, Haemotococcus pluvialis, Thalassiosira pseudonana, Amphidinium* sp., *Symbiodinium microadriaticum,* and *Phaeodactylum tricornurtum*.

Many attempts have been taken to transform *Chlamydomonas reinhardtii* at nuclear, chloroplast and mitochondrial level (Debuchy *et al*., 1989; Boynton *et al*., 1988; Brown *et al*., 1991; Kindle, 1990; Dunahay, 1993; Kumar *et al*., 2004). Foreign DNA can even be introduced into *Chlamydomonas* sp. using simple techniques like glass beads and silicon carbide whiskers because the cell wall is easily permeabilized (Kim *et al*., 2005). However, only three transformation methods, electroporation, PEG (polyethylene glycol) and particle bombardment that are directed at the nuclear level, have been established to transform *Chlorella* sp. (Jarvis and Brown, 1991; Dawson *et al*., 1997; Hawkins and Nakamura, 1999; Chow and Tung, 1999; El-Sheekh, 1999; Chen *et al*., 2001; Kim *et al*., 2002). In general, *Chlorella* can either be transformed by particle bombardment of intact cells or cells after removal of the cell wall, and transformation of the protoplast with PEG-mediated transfection.

2.5.3 Electroporation

Early attempts at the direct gene transfer focussed on enzymatic removal of the cell wall to produce protoplasts. PEG (polyethylene glycol) or electroporation was then used to facilitate DNA uptake through the plasma membrane. Electroporation, which causes temporary pores to be formed in membranes, has also been used to deliver DNA into intact cells, but at low frequencies (Taylor and Fauquet, 2002). It is based upon the induction and stabilization of permeation sites within the cell membrane *via* an interaction of lipid dipoles in an electrical field. The concentrations of both DNA and cells need to be fairly high (approximately 50 µg DNA/ 5 x 10^7 cells), but this ratio has to be optimized for different types of cell. After mixing, the DNA and cell suspension are subjected to three electric pulses at 8 kV cm^{-1} with a pulse decay of 5 µs. However, lower potential square-wave pulse is much favoured because it may increase cell viability (Chu *et al*., 1987; Walker and Rapley, 2000).

2.5.4 Glass Beads with PEG and Silicon Carbide Whiskers Transformation

In glass beads transformation, cells are vortexed in the presence of DNA, glass beads and PEG (polyethylene glycol). This method has been successful used for the transformation of cell-wall deficient mutants or wild type cells of *Chlamydomonas* following enzymatic degradation of the cell wall and is routinely used owing to its simplicity and efficiency (Leon Banares *et al.,* 2004). A similar method using silicon carbide (SiC) whiskers was described for *Chlamydomonas* transformation without removal of cell wall (Dunahay, 1993). Silicon whiskers are microfibers 10 to 80 µm in length and approximately 0.6 µm in diameter. When the target cells, fibers and the desired DNA are vortexed in a liquid medium, the cell wall is wounded and/or penetrated by the needle-like whiskers, allowing the DNA to enter the cell and integrate into the genomic material (Kaeppler *et al*., 1992). SiC-mediated transformation has been used for the genetic manipulation of several higher plants and some microalgae

species such as *Amphinidinium* and *Symbiodinium* (Lohuis and Miller, 1998). According to Taylor and Fauquet (2002), the silicon fiber technology has not been widely used despite being simple and inexpensive. This is probably due to the need to mate the fibers to specific cell types in order to achieve successful transformation and it may be hazardous to health (Taylor and Fauquet, 2002; Leon-Banares *et al*., 2004).

2.6 Promoters

Once the introduced gene has entered the cell and integrated into the host genome, it has to be expressed (Leon-Banares *et al*., 2004). Therefore, it is crucial to develop a suitable promoter system so that the introduced DNA can be satisfactorily expressed by the algal cells. Homologous promoters are usually preferred, since heterologous promoters sometimes do not drive the expression of the transgene in an efficient way (El-Sheekh, 2005). In order to express an introduced gene, a promoter is required to be engineered upstream of this gene (Galun and Breiman, 1998). Not only that, the promoter region has to be adequately recognized by the host's RNA polymerase. Table 2.2 shows the several types of promoters used in the transformation of microalgae. It is generally accepted that in chlorophytes and diatoms, stable expression of heterologous genes can only be optimally achieved when adequate homologous promoters and other regulatory regions are included. However, it is due to the fact that the unique nuclear characteristics of these microalgae that can influence their ability to express genes under the control of heterologous promoters (Leon-Banares *et al*., 2004).

Table 2.2 Promoters used in microalgal constructs (Leon-Banares *et al*., 2004).

Promoters	Gene Sources	Host	Fused Genes	References
Nos	*A. tumefaciens*	*Chlamydomonas* sp., *Amphidinium* sp. and *Symbodinium* sp.	*NptII* and *Nia1* *NptII*	Hall *et al.*, 1993 Lohuis and Miller, 1998
CaMV 35S	Cauliflower mosaic virus	*Chlamydomonas* sp., *Amphidinium* sp. and *Symbodinium* sp.	CAT GUS	Tang *et al.*, 1995 Lohuis and Miller, 1998
Fcp	*Phaeodactylum*	*P. tricornutum*	*Ble* *Glut orHup1* *Ble, NptII, Gfp* or GUS *e-frustulin* *Ble* and *Luc*	Apt *et al.*, 1996 Zaslavskaia *et al.*, 2001 Zaslavskaia *et al.*, 2000 Fischer *et al.*, 1999 Falciatore *et al.*, 1999
RbcS2	*Chlamydomonas*	*Chlamydomonas*	*Gfp* and *Ble* *AphVIII* *Ble* *Cry 1-1* *AadA* *Arg7*cDNA	Fuhrmann *et al.*, 1999 Sizova *et al.*, 2001 Stevens *et al.*, 1996 Nelson and Lefebvre, 1995 Cerutti *et al.*, 1997 Auchincloss *et al.*, 1999
Nia 1 (Nit 1)	*Chlamydomonas*	*Chlamydomonas*	*Ars*	Ohresser *et al.*, 1997 Llamas *et al.*, 2002
Hsp	*Chlamydomonas*	*Chlamydomonas*	*Ars*	Schroda *et al.*, 2002
PsaD	*Chlamydomonas*	*Chlamydomonas*	*Ble, Arg7* or *PsaF*	Fischer and Rochaix, 2001
Cop	*Chlamydomonas*	*Chlamydomonas*	*Gfp* and *Cop*	Fuhrmann *et al.*, 1999
B2-Tubulin	*Chlamydomonas*	*Chlamydomonas*	*AphVII*	Berthold *et al.*, 2002
Acc1	*Cyclotella cryptica*	*C. cryptica* and *Navicula saprophila*	*NptII*	Dunahay *et al*., 1995
p1'2'	*A. tumefaciens*	*Amphinidium* and *Symbiodinium*	*Hpt*	Lohuis and Miller, 1998
CabII-1	*Chlamydomonas*	*Chlamydomonas*	*Nia1*	Blankenship and Kindle, 1990

2.6.1 SV40

The promoter that was used to drive the expression of the HBSAg gene in this research was the SV40 promoter. The fusion plasmid pCAT-HBSAg (6150 bp) had an insertion of a 1.38 Kb sized hepatitis B surface antigen (HbsAg) gene located down stream of the chloroamphenicol acetyl transferase (CAT) gene, which was driven by the SV40 promoter. In 1985, Hasnain *et al*. used SV40 promoter from simian virus to transform *Chlamydomonas*. Teng *et al* (2002), Meng *et al*. (2005) and Steinbrenner and Sandman (2006), successfully applied the SV40 promoter in the transformation of *Haematococcus pluvialis* to transiently express the *lacZ* gene. This promoter was successfully applied to the kelp, *Laminaria japonica* to drive the expression of the hepatitis B surface antigen and the *lacZ* genes (Jiang *et al*., 2002; 2003). In 2003, Gan *et al*. reported that the SV40 promoter could transiently express the *lacZ* gene in the red seaweed, *Gracilaria changii*. According to Qin, *et al*. (2004), SV40 has worked well in *Undaria* for both transient and stable expression. According to Jiang *et al*. (2003), the utilization of SV40 promoter resulted in uniform expression of *lacZ* reporter gene in regenerated *Laminaria* sporophytes, suggesting its high transcription recognition efficiency without histo-specificity.

2.6.2 CAMV35S

The CAMV35S promoter was isolated from CAMV (Cauliflower Mosaic Virus) infected turnip leaves and it can be used to induce the expression of the downstream located coding region. This constitutive promoter is reported to be manifold stronger than the opine promoters and is still one of the most commonly used (Benfey and Chua, 1989). Hawkins and Nakamura (1999) tested several eukaryotic promoters like the CaMV35S, rbcS2 and *Chlorella* Virus promoters to express the human growth hormone in *C. vulgaris* and *C. sorokiniana*. They found that CaMV35S, rbcS2 and *Chlorella* Virus promoter did not differ

distinctly in the expression levels obtained (Hawkins and Nakamura, 1999). Therefore, Hawkins and Nakamura (1999) suggested that the strength of CAMV35s promoter was similar to the rbcS2 and *Chlorella* Virus promoter. Kim *et al.* (2002) reported that the CAMV35S and rbcS2 promoters were active in expressing the flounder growth hormone in *Chlorella ellipsoidea*. Now, CAMV35S have been widely used in *C. vulgaris, C. sorokiniana, C. ellipsoidea, Dunaliella salina and Chlamydomonas reinhardtii* (Kim *et al*., 2002; Hawkins and Nakamura, 1999; Chen *et al*., 2001; Geng *et al*., 2003; Kumar *et al*., 2004). Lohuis and Miller (1998) successfully proved that CAMV35S, heterologous promoter could be used to express the GUS gene in dinoflagellates like *Amphidinium* sp. and *Symbiodinium microadriaticum*. This breakthrough not only proved that dinoflagellates could be transformed and successfully express the introduced DNA in a desired level but also suggested that this promoter might be applicable to other microalgae species (Lohuis and Miller, 1998).

2.6.3 Ubiquitin 1 and *fcp*

The Ubiquitin 1 (Ubi1) promoter is isolated from maize and this non-constitutive promoter activates expression of the introduced genes especially in response to some stresses. This is actually an advantage because the Ubi1 promoter functions efficiently when the target cells are under stress during transformation and selection procedures of transformed target cells (Galun and Breiman, 1998). According to Teng *et al.* (2002), most of the transformation system for *Chlorella* and *Chlamydomonas* mainly employ CaMV35S as a heterologous promoter to control expression. However, Bateman and Purton (2000) suggested that the Ubi1 seemed to be more efficient than the CaMV35S promoter. The addition of a translational enhancer (Ω element) to Ubi1 promoter expressed the highest yield of GUS compared to CAMV35S promoter and the Ubi1 promoter alone (Chen *et al*., 2001).

The *fcp* genes contain the largest number of independent EST sequences, suggesting that it is the most highly expressed gene, and hence it can be used as a strong promoter (Paulsen *et al*., 2006). Steinbrenner and Sandman (2006) reported the transient expression of the *lacZ* gene by the β-carotene ketolase promoter. The *fcp* chlorophyll-a or -c binding protein promoter in diatoms have been used successfully in *Phaeodactylum tricornutum* (Dunahay *et al*., 1995).

2.7 Selection of Selectable Marker Systems and Reporter Genes

According to Galun and Breiman (1998), selectable markers or selectable genes encode proteins that render the transformed cells resistant to phytotoxins which are added to the culture medium, converting it to a selective medium. Selective genes that are used in transformation can be divided into several groups. One group includes genes that confer resistance against antibiotics such as kanamycin, hygromycin and streptomycin. Another group confers resistance against herbicide such as phosphinothricin, biolophos, glyphosphate and dalaphon. The third group is diverse, including genes that cause resistance to high levels of nitrate, amino acid (lysine or threonine) or amino acid analogues. Table 2.3 shows several marker and reporter genes used in microalgal transformation.

Table 2.3 Markers and reporter genes used in microalgal constructs (Leon-Banares *et al*., 2004).

Genes	Descriptions	Type	Gene sources	Host	References
AphvIII	Aminoglycoside 3'--phosphotranferase (resistance to Paromomycin, Kanamycin and Neomycin	Marker	*Stretomyces rimosus*	*Chlamydomonas* sp.	Sizova *et al.*, 2001
Ble	Bleomycin resistance protein (resistance to tallysomycin and related antibiotics)	Marker	*Streptoalloteichus hindustanus*	*Chlamydomonas* sp. *Phaeodactylum tricornutum*	Stevens *et al.*, 1996 Apt *et al.*, 1996
NptII	Neomycin phosphotransferase II (resistance to G418)	Marker	Transposon Tn5 from *Escherichia coli*	*Chlamydomonas* sp. *Amphidinium* sp. and *Symbodinium* sp. *Phaeodactylum tricornutum* *Cyclotella cryptica* and *Navicula saprophila*	Hall *et al.*, 1993 Lohuis and Miller, 1998 Zaslavskaia *et al.*, 2000 Dunahay *et al.*, 1995
Cry1-1	Ribosomal protein S14	Marker	*Chlamydomonas*	*Chlamydomonas*	Nelson and Lefebvre, 1995
Oee-1	Oxygen evolving enhancer protein	Marker	*Chlamydomonas*	*Chlamydomonas*	Chang *et al.*, 2003
CAT	Chloroamphenicol acetyl-transferase (chloroamphenicol resistance)	Marker	Transposon Tn9	*Chlamydomonas*	Tang *et al.*, 1995
Hpt	Hygromycin B phosphotrasnferase	Marker	*Escherichia coli*	*Amphidinium* sp. and *Symbodinium* sp.	Lohuis and Miller, 1998
AadA	Adenyl transferase (spectinomycin resistance)	Marker	Eubacteria	*Chlamydomonas*	Cerutti *et al.*, 1997
Nat	Nourseothricin resistance	Marker	*Streptomyces noursei*	*Phaeodactylum tricornurtum*	Zaslavskaia *et al.*, 2000
Sat-1	Nourseothricin resistance	Marker	*Escherichia coli*	*Phaeodactylum tricornurtum*	Zaslavskaia *et al.*, 2000
e-Gfp	Modified green fluorescent protein	Reporter	Synthetic (adapted to human codon usage)	*Phaeodactylum tricornurtum*	Zaslavskaia *et al.*, 2001
ChGfp	Modified green fluorescent protein	Reporter	Synthetic (adapted to *Chlamydomonas* codon usage)	*Chlamydomonas*	Fuhrmann *et al.*, 1999
GUS	β-Glucuronidase	Reporter	*Escherichia coli*	*Amphidinium* sp. and *Symbodinium* sp. *Phaeodactylum tricornurtum*	Lohuis and Miller, 1998 Zaslavskaia *et al.*, 2000
Glut1	Glucose transporter	Marker or Reporter	Human	*Phaeodactylum tricornurtum*	Zaslavskaia *et al.*, 2001
Hup 1	*Hexose transporter*	Marker or Reporter	*Chlorella kessleri*	*Phaeodactylum tricornurtum* *Cylindrotheca fusiformis*	Zaslavskaia *et al.*, 2001 Fischer *et al.*, 1999
e-frustulin	Calcium-binding glycoprotein	Reporter	*Navicula pelliculosa*	*Cylindrotheca fusiformis*	Fischer *et al.*, 1999
Ars	Arylsulphatase	Reporter	*Chlamydomonas*	*Chlamydomonas*	Ohresser *et al.*, 1997
Luc	Luciferase	Reporter	*Horatia parvula*	*Phaeodactylum tricornurtum*	Falciatore *et al.*, 1999