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A Review on Genetic Engineering of Microalgae with Respect to Genomes, Selectable Marker Genes, Promoters and Reporter Genes

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A B S T R A C T

Genetic engineering of microalgae is emerging at very fast pace because of the potential of this microbe in the field of agriculture, therapeutic recombinant proteins, environment, bioenergy and so on. To work on basic understanding and production of different metabolites from microalgae, information with respect to gene sequences available, promoter, genome, selectable marker, and reporter is required. Nuclear, chloroplastic and other organeller genome information is available online which helps in better understanding of this microalgae. Information with respect to various promoters, selectable markers and reporter genes helps to design different strategies for genetic manipulation of microalgae for production of a variety of value added compounds and fundamental studies of this very promising microorganism.

Introduction

Genetic engineering of algae is an intricate and fast-growing field in biofuel research used for commercial production of fatty acids, pigments, vitamins, proteins, food and feed additives, polysaccharides, cosmetics and biomass (Hallmann 2007). The progress in algal transgenic promises to broaden the existing repertoire of products that have relevance either for medicinal or industrial valuable proteins and/or metabolites by molecular farming. Production of recombinant vaccines, antibodies, bio-hydrogen, insecticidal proteins and so on has been demonstrated in transgenic algae by few authors and the field is still growing (Wang *et al.*, 2012; Gimpel *et al.*, 2015; Rathod *et al.*, 2016). Genetic modifications facilitate to enhance desired physiological chances of algal strains along with few optimization measures in production systems will further improve the prospective of this promising technology in the near future. Normally, single species may not show all the desired traits of interest for commercial production of certain metabolites. Therefore, cautious selection of a suitable target organism and

source of gene should be prioritized at the beginning of every algal transformation experiment (Vira *et al.,* 2016, Hallmann 2007). Several optimization parameters have to be defined such as short life cycle of algae, inexpensive media and high growth rate at different conditions has to be standardized. With respect to these specific characteristics, transformation method needs to be developed or be available for selection of transformed alga (Rathod *et al.,* 2013).

It is also useful to employ an alga in which collections of mutants exist or can be easily generated; but few have their own limitations (Hallmann 2007). Moreover, knowledge on ecological, physiological, molecular or biochemical characteristics of the target species is essential. However, existence of annotated sequence information of algal strains is required for algal transgenic but this is still in its infancy (Kim *et al.,* 2015). The current review focuses on available genome, promoter, selection marker and reporter information in microalgae for designing different molecular biology experiments for fast track development of this emerging field.

Algal genomes

Complete knowledge about the genome sequence is one of the essential features required for genetic manipulations in algae. The genome sequence offers advantages in terms of ease in genetic transformation studies with respect to homology of genes from different sources and site of integration within the genome. Till date, complete genome sequences of more than 20 algal species are available (Gan *et al.,* 2016) the details of which are as shown in Table 1. The genome data is increasing exponentially for algae which are evident from 60 new algal genome projects under progress (Bhattacharya *et al.,* 2015).

Along with nuclear, chloroplast and mitochondrial genomes as discussed, expressed sequence tags (ESTs) also provide the most important sequence information required for molecular genetics and metabolic engineering of microalgae (Gan *et al.,* 2016). ESTs are short, unedited, single pass sequence reads derived from randomly selected complementary DNA (cDNA) libraries. ESTs accelerate gene discovery, complement genome annotation, aid in gene structure identification, establish the viability of alternative transcripts, and facilitate proteome analysis (Hallman, 2007). Recently, there have been advances in sequenced expressed sequence tags (ESTs) among algae, namely, *Chlamydomonas reinhardtii*, *Ostreococcus tauri* and *Acetabularia acetabulum*, the red algae *Porphyra yezoensis*, *Gracilaria gracilis*, *Galdieria sulphuraria*, and so on (Tirichine and Bowler, 2011; Bhattacharya *et al.,* 2015). EST data of algal species are available publicly at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm. nih.gov/projects/dbEST/) and the Taxonomically Broad EST Database (TBestDB, http://amoebidia.bcm.umontreal. ca/pepdb/ searches/welcome.php).

Because of much smaller size of plastid genomes, sequencing of chloroplast and mitochondrial genomes has been performed for more algal species (more than 20) than the genome for EST sequencing projects (Hallman, 2007; Melton *et al.,* 2015). This genome information proves very helpful during chloroplastic or mitochondrial transformation or targeting different proteins to these plastids. The genome sequences for chloroplast and mitochondria are available at the NCBI organelle database (http://www.ncbi.nlm.nih.gov/genomes/ORG ANELLES/ organelles.html) and the Organelle Genome Database (GOBASE, http: //www.bch. umontreal.ca/gobase/gobase.

html). Organelle genome mega sequencing program have been used to generate many organelle sequences (OGMP, http: //www.bch.umontreal.ca /ogmp/) (Hallman, 2007). All the above information will help to answer the phylogenetic questions and to gain a better understanding of the organellar genomic composition (Melton *et al.,* 2015).

Selectable marker genes

Selectable marker genes form an integral part of the transformation process as they assist in selection of transgenic algae. These marker genes are often antibiotic resistance genes and/or auxotrophic mutants with mutations in the corresponding endogenous gene(s). The antibiotic resistant gene is either a dominant or a recessive marker and it confers a new trait to any transformed algal strain. Array of dominant selectable markers are available for algae from a variety of sources which confer antibiotic or herbicide resistance. Table 2 lists markers and their functions in various algal species. Most of the genetic manipulation studies have been carried out in model microalgae *C. reinhardtii* and the maximum numbers of selectable marker genes have been established for this alga. Few examples are discussed in here such as the *aminoglycoside phosphortransferase* (*aph7*) gene from *Streptomyces hygroscopicus* confers resistance against the herbicide hygromycin B (Berthold *et al.,* 2002). The mutated *acetolactate synthase* (*ALS*) gene from *C. reinhardtii* provides tolerance to sulfonylurea herbicides (Kovar *et al.,* 2002). The *aminoglycoside phosphotransferase aphVIII* (*aphH*) gene from *Streptomyces rimosus* provides resistance against paromomycin (Sizova *et al.,* 2001). The mutated *protoporphyrinogen oxidase* (*PPX1*) gene from *C. reinhardtii* confers resistance towards N-phenyl heterocyclic herbicide S-23142 (Randolph-Anderson *et al.,* 1998). The synthetic aminoglycoside adenyltransferase (*aadA*) gene from R100.1 plasmid/bacteriophage T4/ confers resistance to spectinomycin and streptomycin (Cerutti *et al.,* 1997). *Ble* gene from *Streptoalloteichus hindustanus* confers resistance towards zeomycin and phleomycin (Stevens *et al.,* 1996). The mutated ribosomal protein S14 (*CRY1*) gene of *C. reinhardtii* provides resistance to emetine and cryptopleurine (Nelson *et al.,* 1994).

In *H. pluvialis,* the modified gene, *phytoene desaturase* (*pdsMod4.1*), confers resistance to the bleaching herbicide norflurazon by accelerated astaxanthin biosynthesis (Steinbrenner and Sandmann, 2006). In *C. vulgaris,* similar to *C. reinhardtii, the Streptomyces hygroscopicus aminoglycoside phosphotransferase* gene provided resistance against *hygromycin* (Chow and Tung, 1999) with CaMV35S promoter which is usually used in transgenic higher plants. In *L. japonica,* hygromycin resistance was acquired by a simian virus 40 (SV40) promoter-*hygromycin phosphotransferase* chimeric gene (Qin *et al.,* 1999). The SV40 virus, found in humans and monkeys, is a quite surprising promoter that works in brown alga *L. japonica* (Qin *et al.,* 1999).

In diatom *P. tricornutum,* various genes produce antibiotic resistance against different antibiotics. The neomycin *phosphotransferase II (nptII)* gene provides resistance against aminoglycoside antibiotic G418 (Zaslavskaia *et al.,* 2000) whereas the *nat* and *sat-1* genes confer resistance against antibiotic nourseothricin (Zaslavskaia *et al.,* 2000). The *Strepto-alloteichus hindustanus ble* gene was shown to produce resistance against zeomycin (Apt *et al.,* 1996; Falciatore *et al.,* 1999) and the expressed *chloramphenicol acetyltransferase* gene (CAT) confers resistance against the antibiotic chloramphenicol (Apt *et al.,* 1996).

Table.1 List of completed genome sequences of algae

Type of genome	Algae species	GenBank Number	Reference
Nuclear	*Chlamydomonas reinhardtii*	ABCN00000000	Merchant *et al.*, 2007
	Chlorella variabilis	ADIC01000000	Blanc *et al.*, 2010
	Micromonas pusilla	ACCP00000000	Worden *et al.*, 2009
	Ostreococcus lucimarinus	CP000581-CP000601	Palenik *et al.*, 2007
	Ostreococcus tauri	CR954201-CR954220	Derelle *et al.*, 2006
	Volvox carteri f. nagariensis	ACJH00000000	Prochnik *et al.*, 2010
	Nannochloropsis gaditana	AGNI00000000.1	Radakovits *et al.*, 2012
	Nannochloropsis oceanic	AFGK00000000.1	Pan *et al.*, 2011
	Thalassiosira oceanic	GU323224.1	Lommer *et al.*, 2012
	Cyanidioschyzon merolae	AP006483(DDBJ)	Matsuzaki *et al.*, 2004
	Galdieria sulphuraria	ADNM00000000.2	Muravenko *et al.*, 2001; Barbier *et al.*, 2005
	Emiliania huxleyi	AHAL0000000	Read *et al.*, 2013
	Parachlorella kessleri	BBXU00000000.1	Ota *et al.*, 2016
Chloroplast	*Phaeodactylum tricornutum*	EF067920	Oudot-Le Secq *et al.*, 2007
	C. reinhardtii	BK000554	Maul *et al.*, 2002
	Chlorella vulgaris	AB001684	Wakasugi *et al.*, 1997
	P. kessleri	FJ968741	Turmel *et al.*, 2009
	Cryptomonas paramecium	GQ358203	Donaher *et al.*, 2009
	Rhodomonas salina	EF508371	Khan *et al.*, 2007
	E. huxleyi	AY741371	Puerta *et al.*, 2005
Mitochondrion	*C. reinhardtii*	U03843	Maul *et al.*, 2002
	Dunaliella salina	CCAP19/18 – GQ250045	Smith *et al.*, 2010

Table.2 List of selectable markers in algae

Algal culture	Gene for antibiotic resistance	Resistance against antibiotic/herbicide	Reference/s
C. reinhardtii	*Aminoglycoside phosphortransferase*	Hygromycin B	Berthold *et al.*, 2002
	Acetolactate synthase	Sulfonylurea	Kovar *et al.*, 2002
	Aminoglycoside phosphotransferase	Paromomycin	Sizova *et al.*, 2001
	Protoporphyrinogen oxidase	N-phenyl heterocyclic herbicide S-23142	Randolph-Anderson *et al.*, 1998
	Aminoglycoside adenyltransferase	Spectinomycin and Streptomycin	Cerutti *et al.*, 1997
	Strepto-alloteichus hindustanus (*ble*)	Zeomycin and Phleomycin	Stevens *et al.*, 1996
	Mutated *ribosomal protein S14*	Emetine and Cryptopleurine	Nelson *et al.*, 1994
Haematococcus pluvialis	*Phytoene desaturase*	Norflurazon	Steinbrenner and Sandmann, 2006
C. vulgaris	*Aminoglycoside phosphotransferase*	Hygromycin	Chow and Tung, 1999
Dunaliella bardawil	*Hygromycin phosphotransferase*	Hygromycin	Anila *et al.*, 2011
Schizochytrium	*neomycin phosphotransferase II*	G418	Cheng *et al.*, 2012
Laminaria japonica	*Hygromycin phosphotransferase*	Hygromycin	Qin *et al.*, 1999
Phaeodactylum tricornutum	*Neomycin phosphotransferase II*	G418	Zaslavskaia *et al.*, 2000
	Nat and Sat-1	Nourseothricin	Zaslavskaia *et al.*, 2000
	Streptoalloteichus hindustanus (*ble*)	Zeomycin	Apt *et al.*, 1996; Falciatore *et al.*, 1999
	Chloramphenicol acetyltransferase	Chloramphenicol	Apt *et al.*, 1996
Cylindrotheca fusiformis	*Streptoalloteichus hindustanus* (*ble*)	Zeomycin	Fischer *et al.*, 1999
Navicula saprophila	*Neomycin phosphotransferase II*	G418	Dunahay *et al.*, 1995
Cyclotella cryptica	*Neomycin phosphotransferase II*	G418	Dunahay *et al.*, 1995
Symbiodinium microadriaticum	*Hygromycin phosphotransferase*	Hygromycin	Te Lohuis and Miller, 1998
Amphidinium sp.	*Neomycin phosphotransferase II*	G418	Te Lohuis and Miller, 1998

In another diatom, *C. fusiformis*, expression of the *Streptoalloteichus hindustanus ble* gene confers resistance to *zeomycin* under the control of endogenous calcium-binding glycoprotein α-frustulin fruα3 promoter (Fischer *et al.,* 1999). Likewise, in the diatoms *N. saprophila* and *C. cryptic*, the *nptII* gene confers resistance to the antibiotic G418 (Dunahay *et al.,* 1995). The transformation of *S. microadriaticum* and dinoflagellates *Amphidinium sp.* was shown by using the *nptII* gene, or the *hygromycin B phosphotransferase* gene *(hpt)* (Te Lohuis and Miller, 1998). Thus, marker genes play essential role based on specific species used for transformation studies. Besides these dominant selection markers, there is another category which is based on recessive markers in algae. The advantage of recessive markers is that a complete endogenous gene with its own promoter is applicable, though they require auxotrophic mutants with mutations in the corresponding endogenous gene.

The expression and function of recessive selectable markers is quite certain in the respective organism. For example, in *C. reinhardtii*, *Dunaliella viridis*, *Chlorella sorokiniana*, *Volvox carteri*, and *Ulva lactuca*, a very common recessive marker is the *nitrate reductase* (*nit*) gene. It is used for functional complementation of *nitrate reductase* defective mutants (Kindle *et al.,* 1989; Sun *et al.,* 2006; Dawson *et al.,* 1997; Schiedlmeier *et al.,* 1994; Huang *et al.,* 1996). Similarly, *argininosuccinate lyase* (*ASL*) gene was shown to complement mutations in argininosuccinate lyase defective *C. reinhardtii* mutants by selection on arginine-free medium (Debuchy *et al.,* 1989). Studies in *Cyanidioschyzon merolae* show that the wild-type *UMP synthase* gene complements mutated *C. merolae UMP synthase* gene for uracil prototrophy (Minoda *et al.,* 2004). Thus, these wide ranges of selectable markers can act as a basis for selection of transformants while optimizing transformation of a novel algal strain.

Promoter and reporter genes

Promoters could drive the expression of the antibiotic resistance genes i.e. foreign or indigenous genes as well as the reporter genes. Selection of promoter is another vital step for gene transcription process. In most of the cases, selectable marker genes are not able to express by using their own indigenous promoter, especially if the gene source is from a different organism (Hallmann, 2007). However, to address this problem, usually strong, constitutive and/or inducible promoters are used. For higher expression, endogenous promoters are also used as selectable markers or other chimeric gene constructs (Hallmann, 2007).

In algae, various promoters derived from different organisms have been used. For example, CaMV 35S and SV40 promoters from viruses have been used to express target genes in algae (Benfey *et al.*, 1990; Wang *et al.,* 2010). However, the algal genes which usually expresses highly, provide the most effective promoters for gene expression. For example, *Chlamydomonas* heat shock protein 70A gene *hsp70A* (Schroda *et al.,* 2000) and *5′* untranslated region of the *C. reinhardtii* RuBisCO small subunit (*rbcS2*) gene (Stevens *et al.,* 1996), are widely used promoters in *Chlamydomonas* transformation. Reports demonstrate the use of promoters of duplicated carbonicanhydrase1 (DCA1) in *Dunaliella sp.* (Li *et al.,* 2010; Lu *et al.,* 2011), fucoxanthin-chlorophyll a/c binding protein (*fcp*) gene in marine diatom (Apt *et al.,* 1996; Miyagawa-Yamaguchi *et al.,* 2011), actin1 (PyAct1) gene in *Porphyra yezoensis* (Takahashi *et al.,* 2010), and violaxanthin/ chlorophyll a-binding protein (VCP) genes in *Nannochloropsis* sp. (Kilian *et al.,* 2011). Perhaps, for the confirmation of

transformation event and its visual detection and identification of expressed reporter protein, different genes are extensively demonstrated in algae. Reporter genes usually code for fluorescent protein, enzymes that convert a colorless substrate into a colored product, or result in light emission. For example, endogenous *arylsulfatase* (*ARS)* gene is a valuable reporter gene which converts chromogenic substrates like 5-bromo-4-chloro-3-indolyl sulphate (X-SO_4) or p-nitrophenyl sulfate into color compound allowing analysis, both at subcellular localization as well as spectrophotometric quantification of arylsulfatase activity (Davies *et al.,* 1992; Hallmann and Sumper, 1994).

A codon-optimized *green fluorescent protein (GFP)* gene which does not require any additives for GFP detection, can be visualize using fluorescent microscope (Fuhrmann *et al.,* 1999; Ender *et al.,* 2002). Examples of other reporter genes are the codon-optimized *Renilla reniformis luciferase (crluc)* gene in *C. reinhardtii*, or the *Chlorella kessleri* hexose/H+ symporter (HUP1) gene in *Volvox carteri* (Fuhrmann *et al.,* 2004; Hallmann, 2007).

Escherichia coli β-glucuronidase (GUS) *uidA* reporter gene has also been used successfully in several algal species such as *D. salina*, *Porphyra yezoensis*, *Thalassiosira weissflogii*, *C. fusiformis*, *C. kessleri*, *C. vulgaris*, and *Porphyra miniata* where different β-glucuronidase substrates, like 4-methylumbelliferyl-β-D-glucuronide (MUG), 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) or p-nitrophenyl β-D-glucuronide help to analyze protein expression, both at subcellular localization level and spectrophotometry quantification (Tan *et al.,* 2005; Cheney *et al.,* 2001; Falciatore *et al.,* 1999; El-Sheekh, 1999; Chow and Tung, 1999; Hallmann, 2007).

In *Chlorella ellipsoidea* and *P. tricornutum*, firefly *luciferase* gene was used as a reporter system (Jarvis and Brown, 1991; Falciatore *et al.,* 1999); and the expression of luciferase is detected by *in-vitro* imaging. Another *Escherichia coli β-galactosidase (lacZ)* gene was shown to be an important reporter gene for *Gracilaria changii*, which requires *in-vitro* application of the substrate 5-bromo-4-chloro-3-indolyl-β- D-galactoside (X-gal) (Hallmann, 2007). In diatoms, *T. weissflogii* and *C. fusiformis*, the *GFP* gene, was used as reporter gene (Poulsen and Kröger, 2005). In *P. tricornutum* and *V. carteri*, overexpression of a gene encoding glucose transporters (glut1 or HUP1), allows these algae to grow in dark on exogenous glucose (Hallmann, 2007). These reporter genes can be used to assist in successful transformation phenomenon of algae.

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