CHAPTER 1

Nuclear Transformation of Eukaryotic Microalgae

Historical Overview, Achievements and Problems

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Abstract

Transformation of microalgae is a first step in their use for biotechnological applications involving foreign protein production or molecular modifications of specific cell metabolic pathways. Since the first reliable achievements of nuclear transformation in *Chlamydomonas*, other eukaryotic microalgae have become transformed with molecular markers that allow a direct selection. Different methods—glass beads, electroporation, particle bombardment, or Agrobacterium—and constructions have been set up in several organisms and successfully used. However, some problems associated with efficiency, integration, or stability of the transgenes still persist and are analysed herein. Though the number of microalgae species successfully transformed is not very high, prospects for transformation of many more are good enough on the basis of what has been achieved so far.

Introduction

Microalgae constitute a highly heterogeneous group of prokaryotic and eukaryotic organisms with a capital ecological importance, accounting for about 50% of the global organic carbon fixation[1] and having an enormous biotechnological potential. Many species have been used for the production of high-added-value compounds of application in feeding, dietetics, cosmetics and fine chemistry industries; and in various processes such as wastewater treatment or biofertilization.[2-4] Furthermore, microalgae are used as model systems for studying fundamental processes such as photosynthesis, flagellar function, photoreception and nutrient acquisition[5,6] and have been proposed as an alternative system for the expression of heterologous proteins, including antibodies and other therapeutic proteins.[7,8]

In contrast to the large number of genetically modified bacteria, yeast and even higher plants, only a few species of eukaryotic microalgae have been successfully transformed with a certain efficiency. The development of molecular tools for efficient and stable genetic manipulation of microalgae is therefore necessary to enhance their potential for engineering their metabolic pathways.

The best studied genetically modified eukaryotic microalga is so far the freshwater chlorophyte *Chlamydomonas reinhartii*, which was first transformed in 1989 by complementation of *nit1* and *arg7* mutations with homologous nitrate reductase[9] and argininosuccinate lyase genes,[10] respectively. Since then a significant number of selectable markers, promoters, and new procedures

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Table 1. Summary of the main species of microalgae genetically modified and the transformation method used

Division	Species	Method	Ref.
Dinoflagellates	Amphidinium	Silicon carbide whiskers	37
	Symbiodinium	Silicon carbide whiskers	37
Diatoms	*Phaeodactylum tricornutum*	Bombardment	36,32
	Cyclotella criptica	Bombardment	34
	Navicula saprophila	Bombardment	34
	Cylindrotheca fusiformis	Bombardment	35
	Thalassiosira weissflogii	Bombardment	36
Chlorophyceae	*Chlamydomonas*	Glass beds	39
		Electroporation	42
		Silicon carbide whiskers	41
		Bombardment	10,44
		Agrobacterium	45
	*Chlorella ellipsoidea**	Bombardment	19
		Electroporation	23
	Chlorella saccharophila	Electroporation	20
	*Chlorella vulgaris**	Protoplast transformation	21
	*Haematococcus pluvialis**	Bombardment	26
	Dunaliella salina	Electroporation	29,27
		Bombardment	28,30

* In some cases only transient expression has been observed.

for efficient introduction of DNA into microalgal nucleus have been developed and transformation efficiency has dramatically increased. Nevertheless the number of transformed species has timidly increased to about a dozen of new strains (Table 1).

Excellent reviews on different aspects of genetic transformation of microalgae,[11-13] some focused exclusively on *Chlamydomonas*,[14,-16] have been previously published. Here, we will review the main methods and strategies presently used for nuclear transformation of eukaryotic microalgae, including those species that have been successfully transformed and discuss the main difficulties associated with stable expression of transgenes. The transformation of chloroplast and cyanobacteria has specific characteristics that are treated in Chapters 4 and 2, respectively.

Microalgae Groups Transformed

Microalgae are phylogenetically very heterogeneous. Until now, there are reports of stable nuclear transformations in three eukaryotic microalgal groups: Chlorophytes, diatoms, and dinoflagellates (Table 1).

Chlorophytes

The freshwater alga *Chlamydomonas* is the first and best studied transformed chlorophyte[14,15,16] that has already become a powerful model system for molecular studies[5,6,17,18] due to its easy manipulation techniques and the availability of bioinformatic tools, such as an EST database (http://www.chlamy.org) and a draft of the complete genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). A large variety of transformation methods and constructions have been designed for this microalga (see Table 2) and several biotechnological processes involving transgenic *Chlamydomonas* have been described. Production of H_2 (see Chapter 10), recombinant vaccines (Chapter 11) and bioremediation (Chapter 9) are some examples.

Table 2. Examples of marker and reporter genes used in microalgal constructs

Gene	Description	Gene Source	Ref.
aadA	Adenylyl transferase (resistance to spectinomycin)	*Eubacteria*	74
als	Acetolactate synthase (resistance to sulfonylurea herbicides)	*Chlamydomonas*	70
aphVIII	Aminoglycoside 3'phosphotransferase (resistance to paramomycin)	*Streptomyces rimosus*	69
ars	Arylsulphatase	*Chlamydomonas*	59
ble	Bleomycin binding protein (resistance to zeocin)	*Streptoalloteichus hindustanus*	*83*
cat	Chloramphenicol acetyltransferase (resistance to chloramphenicol)	Transposon Tn9	66
cry1-1	Ribosomal protein S14	*Chlamydomonas*	52
ε-frustulin	Calcium binding glycoprotein	*Navicula pelliculosa*	35
gfp^{c}	Modified green fluorescent protein	Adapted to *Chlamydomonas* codon usage	57
gfp^{e}	Modified green fluorescent protein	Adapted to human codon usage	33
glut1	Glucose transporter	Human	84
gus	β-glucoronidase	*Escherichia coli*	37
hpt	Hygromycin B phosphotransferase	*Escherichia coli*	37
hup1	Hexose transporter	*Chlorella kessleri*	84
luc	Luciferase	*Horatia parvula* (firefly)	51
nat	Nourseothricin resistance	*Streptomyces noursei*	33
nptII	Neomycin phosphotransferase II (resistance to G418)	*Escherichia coli*	*64*
oee-1	Oxygen evolving enhancer protein	*Chlamydomonas*	85
sat-1	Nourseothricin resistance	*Escherichia coli*	33

The first successful introduction and expression of foreign DNA in *Chlorella* cells reports on the transient expression of firefly luciferase (*Luc*) in protoplasts of *C. ellipsoidea*[19] and of β-glucuronidase (*Gus*) gene in *C. saccharophila* by electroporation.[20] The expression of the human growth hormone (*hGH*) under the control of different promoters including a *Chlorella* virus promoter, the RbcS2 promoter from *Chlamydomonas reinhardtii* and the CaMV35S promoter was also achieved in *C. vulgaris* and *Chlorella sorokiniana* protoplasts.[21] Although expression was not stable for more than a couple of months, these authors were able to induce excretion of the heterologous protein by inserting an extracellular secretion signal sequence between the promoter and the *hGH* gene. The first evidence of stable transformation of *Chlorella* was the complementation of nitrate reductase deficient mutants of *C. sorokiniana* with a homologous gene of nitrate reductase from *C. vulgaris* by microprojectile bombardment that was reported as an event of homologous recombination.[22] Difficulties for getting stable transgene integration and expression in *Chlorella* transformants will require further studies to ascertain the relevance of homologous integration. Anyway, expression of commercially interesting genes such as the heterologous rabbit neutrophil peptide-1(NP-1),[23] the flounder growth hormone,[24] and a bio-insecticide-acting peptide hormone from mosquito ovaries[25] has been reported in *Chlorella* though stability of the expression of these genes is not clearly stated.

Other chlorophytes with important economic value, such as *Dunaliella* or *Haematococcus*, have been for a long time refractory to any type of genetic manipulation, and only very recently some promising reports of successful transformation have arisen. Transient expression of β-galactosidase in *Haematococcus pluvialis* by bombarding the *LacZ* gene driven by the SV40

promoter,[26] and integration into *Dunaliella* genome of foreign genes by electroporation[27] or micro-particle bombardment[28] were described. However, stable foreign gene expression has only recently been announced.[29,30] The lack of specific promoters for these species is the main limitation for the development of efficient and stable transformation systems. The flanking regions of *Dunaliella rbcS2* gene were used to drive the expression of marker genes in *C. reinhardtii,*[31] opening the way for the efficient genetic manipulation of this halophilic microalga, which is the main source of natural beta-carotene.

Diatoms

Several strains in this group have important biotechnological applications derived from their silicean cell wall and their use in aquaculture and as source of particular oils[2] (Apt and Behrens, 1999). Their genetic manipulation and biotechnological applications are presented in Chapter 3. *Phaedactylum tricornutum,*[32,33] *Cyclotella criptica,*[34] *Navicula saprophila,*[34] *Cylindrotheca fusiformis,*[35] and *Thalassiosira weissflogii*[36] are some of the strains successfully transformed.

Dinoflagellates

Dinoflagellates or pyrrophyta are a group of unicellular eukaryotic alveolar algae that constitute an important part of marine phytoplankton. Some species of this division produce luminescent compounds; others produce compounds that are toxic to vertebrates including humans. Species of this group have typical eukaryotic cytoplasmatic features but the characteristics of their nucleus are relatively unique. They lack histones and combine typical prokaryote characteristics, such as permanently condensed chromosomes, with eukaryotic features, such as the presence of nucleolus and introns. The strains *Amphidinium* and *Symbiodinium* have been successfully transformed.[37] In both cases intact walled cells were transformed by agitation in the presence of silicon carbide whiskers. The *Gus* gene, fused to neomycin phosphotransferase (*nptII*) or hygromycin phosphotransferase (*hpt*) genes, that confer resistance to kanamycin or hygromycin were expressed under the control of either *Agrobacterium* p1'2'promoter or cauliflower mosaic virus 35S promoter. The unique nuclear characteristics of these microalgae can influence their ability to express genes driven by heterologous promoters. Although prolonged selection is required, dinoflagellate genera *Amphidinium* and *Symbiodinium* might be considered as one of the most accessible groups of algae for genetic manipulation.

Others

The scope of this review does not include multicelullar agae, although it should be noted that efficient transformation systems have been developed in species such as *Volvox cateri,*[38] the carrageenan-producing rhodophyte *Kappaphycus alvarezii* or the green macroalga *Ulva lactuca.*[11]

Methods for Microalgae Transformation

A variety of alternative approaches for gene transfer to eukaryotic microalgal nucleus have been developed. The basis of the traditional methods used to transform microalgae is to cause, by different means, a temporal permeabilization of the cell membrane, enabling DNA molecules to enter the cell while preserving viability.

Agitation in the Presence of Glass Beads or Silicon Carbide Whiskers

In the glass beads method, permeabilization of the cells is obtained by vortexing in the presence of DNA, glass beads and PEG.[39] It was originally developed for yeasts and has been successfully used for transformation of cell-wall deficient mutants or wild-type cells of *Chlamydomonas* following enzymatic degradation of the cell wall. No reports about the efficiency of this method for transforming other species exist, but the simplicity and efficiency of this procedure make it very interesting to investigate whether it can be applied to other species of microalgae. The main advantage of this method is that it does not require specialized equipment. Though the

main drawback is considered to be the requirement of cell wall-less strains as hosts, low efficient transformations are achieved even with intact walled *Chlamydomonas* cells.[40]

Dunahay[41] (1993) described a similar method using agitation with silicon carbide whiskers for *Chlamydomonas* transformation without removing of the cell wall. This method has also been successfully used for the transformation of the dinoflagellates, *Amphidinium* and *Symbiodinium.*[37]

Electroporation

In electroporation, transient holes in the cell membrane are formed when pulses of an electric current are applied. The introduction of genes by electroporation has been widely carried out in animal, plant and bacterial cells. Stable transformants of both wall-less and walled strains of the microalga *Chlamydomonas reinhardtii,*[42] *Chlorella ellipsoidea,*[23] and *Dunaliella salina*[29] have been obtained by this method. Temperature, osmolarity, electric conditions, field strength ($kVcm^{-1}$), time of discharge, and DNA concentrations have to be carefully optimised to obtain high transformation efficiencies. High efficiency of transformation is achieved in *Chlamydomonas* to 2×10^{-5} transformants per cell and μg of DNA, about two orders of magnitude higher than that obtained with the standard glass beads method to introduce exogenous DNA.[42]

Microparticle Bombardment

In the microparticle bombardment or biolistic method the DNA is literally shot into the host cells with a "gene gun". Small gold or tungsted particles (0.5-1.5 μm) are coated by DNA and accelerated (at about 500m s^{-1}) by an Helium driven gun into the target cells. It has been mainly applied for transformation of plant cells and tissues[43] but it has also been successfully used for transformation of other biological systems as diverse as animal cells tissues, fungi, subcelullar organella, bacteria and algae. The technique has demonstrated to be particularly successful for transformation of microalgae that are refractory to other transformation methods, such as diatoms[32,34] or walled clorophytes.[44]

Agrobacterium tumefaciens

Agrobacterium tumefaciens is a common tool in plant genetic transformation because it has evolved the unique capacity to transfer a piece of its own DNA (the T-DNA) from the Ti plasmid into the nuclear genome of plant cells. This represents an example of naturally occurring trans-kingdom transfer of genetic material and has enabled this bacterium to be used as a tool to integrate selected genes in plant chromosomes. The method was initially applied to dicotyledonous plants, which are the natural host for this pathogenic soil bacteria, but it was soon used for genetic manipulation of monocotyledonous plants, including cereals, and fungi. The stable genetic transformation of the unicellular microalgae *C. reinhardtii* by *Agrobacterium* has recently been reported.[45] These authors cocultivated *Chlamydomonas* and *Agrobacterium* cells carrying a plasmid which contains the hygromycin phosphotransferase *(hpt)* gene and the gene fusion *gfp:uidA* as reporter gene, both driven by the CaMV 35S promoter. Transformation efficiency obtained by this method is 50-fold higher than that of the glass beads transformation. Until now no other reports on microalgae transformation using this method have been published. However the very wide host range of *Agrobacterium,* including gymnosperms and perhaps lower plant phyla, a variety of fungi, and even animal cells, suggests that T-DNA transfer might become a common system to deliver genes in algae.[46]

Other Methods

Other approaches, such as the use of recombinant viruses as vehicles to carry foreign DNA into algae, the utilization of transposons elements or microinjection are being studied. There are early reports of transformation by microinjection of the giant (2-4cm) algae *Acetabularia*[47] which is unicellular for most of its lifecycle. But application of this method to usual microscopic microalgae has obvious size limitations.

Several viruses infecting *Chlorella* and brown algae are being considered for developing both cloning vehicles and expression systems. A *Chlorella* virus promoter sequence was successfully used by Hawkins and Nakamura[21] (1999) for transient expression of a gene encoding for human growth hormone (*hGH*). Reciprocally, a *Chlorella* virus adenine methyltransferase gene promoter functions as a strong promoter in plants, and is significantly stronger than the cauliflower mosaic virus 35S promoter.[48] The large size (150-330 kpb) of the algal viral genomes may permit insertion of foreign genes and provide mechanisms for directing their insertion into algal chromosomes,[49,50] but more extensive studies about eukaryotic algal viruses are necessary before this exciting approach became a reality.

Characteristics of the Transformation Process

In contrast to prokaryotes or organelles, foreign DNA preferently integrates at apparently random locations of the eukaryotic nuclear genome by nonhomologous recombination, difficulting the deletion or repairing of the selected genes of interest.[51] This obstacle has been exploited as a method for insertional mutagenesis, whose fundamentals and applications are addressed in Chapter 7. Homologous recombination in nuclear genes was observed at low frequency in *Chlamydomonas*,[52,53] and recently this frequency has been increased by two orders of magnitude by using single stranded DNA.[54]

The number of copies of the introduced DNA varies from one to several depending on the DNA concentration and the transformation technique.[5] It has been described in *Chlamydomonas* that by using low concentrations of DNA (about 100 ng or less per 10^8 cells) most integrations occur with a single copy of marker DNA.[55] In transformants bearing multiple copies of the marker, these are often integrated as concatamers of transforming DNA.[44] This is expected from the amount of DNA (1 μg and higher) routinely used in transformations. The transformation with linearized DNA seems to be more efficient than with circular DNA, and intregration events are more predictable. Nevertheless, it is important that the chosen cleavage site is not very close to the 5′ or 3′ UTR regions of the introduced gene to preserve its integrity.[56]

One of the difficulties found to express heterologous genes in microalgae is the selection of transformants. Cotransformation with two different plasmids, one of which contains an easily selectable marker, can solve the problem, although frequency of expression of both genes can be low (10-50 %). This efficiency increases dramatically if the two genes are within the same construction.[57]

DNA Constructions Used in Transformation

Availability of marker and reporter genes is of key importance for selection of the transformed microalgae. For several years, the only adequate selectable markers available for microalgae transformation were based on homologous genes (*arg7*, *nia1=nit1*, *oee1* or *atpC*) that allowed complementation of specific mutants. But this strategy is not applicable to wild type or diploid microalgae (i.e., diatoms) owing to the difficulties to generate mutants in which both alleles of a gene are defective. Nowadays, a large collection of reporter genes and selectable markers are available, some of which are summarized in Table 2.

The most powerful selectable markers are those that confer resistance against antibiotics such as bleomycine (*Ble*), streptomycin (*aadA*) and paramoycin (*aphVIII*) or to herbicides, such as the gene Acetolactate synthase (*als*) that confers resistance to sulphonylurea herbicides. Some reporter genes as *Gus* (β-Glucuronidase) and adapted versions of *GFP* could also be used to test protein subcellular localization when fused to the desired protein.[16,58] The *Luc* gene, that codes for luciferase of *Renilla reniformis* adapted to *C. reihardtii* nuclear codon usage, expressed under the control of different promoters has been used for "in vivo" monitoring nuclear gene expression.[51] The *ars* gene also provides an easy and rapid assay for promoter activity studies, as shown in *Chlamydomonas*.[59,60]

The choice of highly active promoters to drive the expression of marker and reporter genes is a critical step in the development of an efficient transformation system. Strong constitutive

Table 3. Selected constructs used for microalgal transformation

Microalgal Host	Promoter	Fused Gene	Ref.
Chlamydomonas	*nia1* (Nitrate reductase from *C. reinhardtii*)	*ars*	59,60,63
"	*rbcS2* (Rubisco small subunit from *C. reihardtii*)	*gfp+ble*	57
"	"	*aphVIII*	67
"	"	*ble*	83
"	"	*aadA*	74
"	"	*als*	70
"	*hsp70A* (Heat shock protein from *C. reinhardtii*)	*ars*	61
"	*psaD* (Photosystem I complex protein from *C. reinhardtii*)	*ble*	71
"	*cop* (Chlamyopsin from *C. reinhardii*)	*gfp+cop*	57
"	*nos* (Nopaline synthase from *A. tumefaciens*)	*nptII+nia1*	45
"	*CaMV35S* (Cauliflower mosaic virus 35S)	*Cat*	66
Chlorella	*ubi1-Ω* (ubiquitin-Ω from *Zea mais*)	*Gus*	23
Haematococcus	*SV40* (simian virus 40)	*LacZ*	26
Dunaliella	*ubi1-W* (ubiquitin-Ω from *Zea mais*)	*Gus*	29
Phaeodactylum tricornutum	*fcp* (Fucoxanthin chlorophyll-a or -c binding protein)	*Ble*	32
"	"	*glut-1/hup1*	84
"	"	*ble/nptII/gfp/gus*	33
"	"	*ε-frustulin*	35
"	"	*ble* and *luc*	36
Amphidinium and Symbiodinium	*nos* (Nopaline synthase from *A. tumefaciens*)	*NptII*	37
	p1'2' (from *A. tumefaciens*)	*hpt*	37

homologous promoters, such as the *rbcS2* gene promoter of *C. reinhardtii* or the *fcp*A and B promoters of *P. tricornutum* are widely used to drive the expression of heterologous proteins. The upstream fusion of a *hsp70A* promoter fragment enhances activity of neighbouring promoters[61] and decreases the probability of transcriptional silencing,[62] which is an important problem in the expression of transgenes. The use of inducible promoters like the nitrate reductase gene promoter of *Chlamydomonas*,[59,60,63] or the promoter derived from the nitrate reductase gene of the diatom *Cylindrotheca fusiformis*,[64] provides a greater control in the expression. Description of selected promoters used to express heterologous proteins in microalgae is shown in Table 3.

Attempts to express genes fused to heterologous promoters in diatoms were unsuccessful.[32] In Chlorophytes, transformation frequency with heterologous constructs was low[65] or expression was unstable.[28,66] The only report of stable transformation of microalgae with heterologous genes under the control of heterologous promoters is the expression of *Gus* driven by either the cauliflower mosaic virus 35S promoter or the p1'2' *Agrobacterium* promoter in dinoflagellates *Amphidinium* and *Symbiodinium*.[37] The unique nuclear characteristics of these microalgae can influence their ability to express genes under the control of heterologous promoters. A certain frequency of transformation has been achieved with promoter-less heterologous genes in *Chlamydomonas*, probably as a result of in vivo gene-fusion with endogenous promoter regions.[56,67] Identification of promoters ('promoter trapping') was achieved in *Chlamydomonas* with arg7 as a selectable marker, and a promoter-less reporter gene (*rsp3*), which was expressed in 2-3% of the transformants.[68] However, it is generally accepted that, in chlorophytes

and diatoms, stable expression of heterologous genes can only be optimally achieved when adequate homologous promoters or promoters from very close species are included.[31,34]

The presence of introns was also shown to be a factor for efficient expression. *Ble* and *aphVIII* genes, under *rbcS2* promoter, increased their expression by including the first intron of *rbcS2*.[69] Expression of the *als* gene under *rbcS2* promoter was also optimal when all *als* introns were present.[70] However, introns are not absolutely required for gene expression as shown with the cDNA of *arg7*.[56] Expression vectors having promoter and *cis* elements from the highly expressed gene *psaD*, lacking introns, are useful to allow high-expression of endogenous and exogenous cDNAs.[71]

Difficulties for Stable Expression of the Transgenes

A great number of regulatory elements and transcriptional or post transcriptional events can influence on the expression level of the transgenes and on their stability. Expression of an exogenous gene can be very low or null, even though all the elements required for optimal transcription and translation -promoters, introns and other regulatory regions- have been included in the chimeric gene construction. Futhermore, when transgenic algal clones are not maintained under selection conditions, expression of the exogenous genes might be suppressed. This need can be avoided in *Chlamydomonas* by freezing transgenic cells in liquid nitrogen for which a protocol has been established (R. Sayre at http://www.chlamy.org/methods/freezing.html and Crutchfield et al[72]) and optimized (http://www.uco.es/organiza/departamentos/bioquimica-biol-mol/english/files/Chlamy_cryopreservation.pdf).

Difficulties for foreign gene expression in microalgae can be due to the lack of adequate regulatory sequences, to positional effects, biased codon usage—that is analysed in Chapter 5—incorrect polyadenylation, inappropriate nuclear transport, unstability of the mRNA, or gene silencing. Gene silencing has been attributed to a variety of epigenetic mechanisms similar to those observed in plants and other eukaryotic cells[73] and is thought to be related to the control of development and to the response of a cell to viruses, transposable elements, or transgenes.[74,75] Epigenetic processes are defined as heritable changes in gene expression without modification of DNA sequences. These changes might occur at the level of transcription or post-transcription by mechanisms that are not yet fully understood. Several elements involved in the silencing of transgenes in *Chlamydomonas* have recently been isolated.[76] Transcriptional gene silencing can occur through an altered chromatin structure that is associated with cytosine methylation of the promoter regions. However, single-copy transgenes can be transcriptionally silenced without detectable cytosine methylation of introduced DNA.[75,77,78] Post-transcriptional transgene silencing can take place by the RNA-mediated process called interference RNA (RNAi). RNAi forms RNA-induced silencing complexes that promote RNA degradation.[75] It is worth noting that mechanistic connections between epigenetic transcriptional silencing and DNA double-strand-break repair have recently been proposed.[78] This disadvantage for gene expression can result in the useful technique of RNA interference (RNAi) that has already helped to the functional characterization of a good number of genes[79] (see Chapter 7).

In addition, chromatin states are determined by Lys methylation of histones mostly mediated by SET domain-containing proteins and depending on the methylated histone residue, the degree of methylation, and the chromatin context, transcriptional repression or activation occurs.[80,81] It has been recently shown that Mut11p, required for transcriptional silencing in *Chlamydomonas reinhardtii*,[78] interacts with conserved components of histone methyltransferase machineries, and proposed that the functional differentiation between dimethylated and monomethylated H3 lysine4 operates as an epigenetic mark for repressed euchromatin.[82] This intricate circuit of methylations might provide methodologies and tools to activate or inhibit, as needed, gene expression in different algal systems.

Concluding Remarks

A speedy progress has been attained since the first reliable transformation techniques in the microalga *Chlamydomonas*. Several methodologies has been developed that primarily rely on an

efficient and reproducible transformation. Though much progress has been achieved in other different microalgae, there is still the need to develop general strategies to introduce DNA stably in any algal system. These may come from understanding more deeply the molecular biology of these systems. Tools like wide-spectrum general promoters, DNA markers, and ssDNA transformation, or *Agrobacterium* might provide future developments. Time will tell us.

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