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Edited by Rosa León, Aurora Gaván, and Emilio Fernández

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CONTENTS

PREFACE

Microalgae have been largely cultured and commercialised as food and feed additives, and their potential as source of high-added value compounds is well known. But, in contrast to the large number of genetically modified bacteria, yeast and even higher plants, only a few species of microalgae have been genetically transformed with efficiency. Initial difficulties in the expression of foreign genes in microalgae have been progressively overcome, and powerful molecular tools for their genetic engineering are now on hand. A considerable collection of promoters and selectable marker genes and an increasing number of genomic or cDNA sequences have become available in recent years. More work is needed to transform new species of microalgae, especially those that have commercial value, so that it would be possible to increase the productivity of traditional compounds or synthesize novel ones. Silencing transgenes remains an important limitation for stable expression of foreign genes. This problem is not unique to microalgae since it has also been observed in plants, animals and fungi. A better understanding of the mechanisms that control the regulation of gene expression in eukaryotes is therefore needed.

In this book a group of outstanding researchers working on different areas of microalgae biotechnology offer a global vision of the genetic manipulation of microalgae and their applications. In Chapter 1 the transformation methods and some of the problems and achievements in the genetic manipulation of microalgae, mostly related to the chlorophyte *Chlamydomonas*, are reviewed from the first successful experiments to date. Further chapters provide an ample view of the genetic manipulation of cyanobacteria (Chapter 2) and diatoms (Chapter 3) are also included. The transformation of chloroplasts is an interesting alternative to the nuclear transformation that allows gene integration by homologous recombination and is studied in detail in Chapter 4. To complete the fundamental aspects of the genetic manipulation of microalgae, the influence of codon usage on the expression of heterologous proteins is analyzed in Chapter 5. Bias in codon usage is one of the main limitations for expression of foreign genes in microalgae that has stimulated the search for creative solutions, such as the synthesis of genes adapted to host codon usage.

The book includes chapters focussing on biotechnological applications of transgenic microalgae. The enormous amount information generated by the genomic or cDNA sequencing projects and its implications on the development of new biotechnological applications of microalgae are analysed in Chapter 6. The non-homologous

recombination in eukaryotic microalgae has made difficult the directed knockout of selected genes, but in turn it has provided an excellent method for mutagenesis and for the construction of tagged collections of mutants (Chapter 7). Pioneering works about the use of transgenic microalgae as efficient cell factories for the expression of recombinant proteins (Chapter 8), bioremediation (Chapter 9), production of hydrogen (Chapter 10), and recombinant vaccines (Chapter 11) are also included.

We are grateful to the contributing authors for their excellent work. They reviewed the published research work in their area of expertise and shared their unpublished results to bring the chapters up to date. Editing this book on the back of other research and academic duties has been demanding of our time, and we thank family and friends for their tolerance.

Finally, we do hope that the book will prove useful for algologists, biotechnologists, and researchers interested in green cell factories.

Rosa León, Ph.D.
Aurora Galván, Ph.D.
Emilio Fernández, Ph.D.

ABBREVIATIONS

BBS	Bardet-Biedl syndrome
BKD	bacterial kidney disease
Ble	phleomycin resistance marker
C4	carbon fixation through 4 carbon intermediates
CCM	carbon concentrating mechanism
CCMP	Center for Culture of Marine Phytoplankton
cDNA	copy DNA
CFP	cyan fluorescent protein
Chl	chlorophyll
Ci	inorganic carbon
CIA5	inorganic carbon activator protein
CMY	heavy metal biosensor
CP57	chloroplast expressed p57 antigen
E22	nuclear expressed membrane fusion protein with C-terminal, 14 amino acid, p57 epitope
ELIP	early light inducible protein
EST	expressed sequence tag
ESV	*Ectocarpus siliculosus* virus
EXAFS	extended X-ray absorption fine-structure spectroscopy
Fd	ferredoxin
Fo	minimal chlorophyll fluorescence yield (observed in very low light and primarily from photosystem II)
FRET	fluorescence resonance energy transfer
GAPC	gut-associated phagocytotic cells
gDW	grams dry weight
GFP	green fluorescent protein
GPA	glycine, proline, alanine-rich protein
HLIP	high light inducible protein
JGI	Joint Genome Institute
kGDW	kilogram dry weight
LHC	light harvesting complex
LHCA	light harvesting protein A (photosystem I)
LHCB	light harvesting protein B (photosystem II)
Lor	loroxanthin (when lower case and italicized it indicates a mutant in loroxanthin synthesis)
Mb	megabasepairs
MT	metallothionein, class II
NP57	nuclear expressed p57 protein
Npq	nonphotochemical quenching (when lower case and italicized it indicates a mutant strain)
NUPT	nuclear integrant of plastid DNA
P	phosphorus
P5CS	pyrroline-5-carboxylate synthase
PBCV	*Paramedium bursaria chlorella* virus
PHOT	phototropin
PS	photosystem
PSR	phosphorus starvation response
RNAi	RNA interference
ROS	reactive oxygen species
SAC	sulfur acclimation
SDV	silica deposition vesicle
SEPs	stress enhanced protein
TEM	transmission electron microscopy
UV	ultraviolet
WT	wild-type
YFP	yellow fluorescent protein

CHAPTER 1

Nuclear Transformation of Eukaryotic Microalgae

Historical Overview, Achievements and Problems

Rosa León* and Emilio Fernández

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.