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Edited by Rosa León, Aurora Gaván, and Emilio Fernández

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Transgenic Microalgae as Green Cell Factories

Edited by

Rosa León, Ph.D.

*Departamento de Química y Ciencia de Materiales, Facultad de Ciencias
Experimentales, Universidad de Huelva, Huelva, Spain*

Aurora Galván, Ph.D.

*Departamento de Bioquímica y Biología Molecular, Campus de Rabanales,
Universidad de Córdoba, Córdoba, Spain*

Emilio Fernández, Ph.D.

*Departamento de Bioquímica y Biología Molecular, Campus de Rabanales,
Universidad de Córdoba, Córdoba, Spain*

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PARTICIPANTS

Pio Colepicolo
Department of Biochemistry
University of Sao Paulo
Sao Paulo
Brazil

Konrad Dabrowski
Department of Natural Resources
Ohio State University
Columbus, Ohio
U.S.A.

Almut Eckert
Kompetenzzentrum
für Fluoreszente Bioanalytik
Universität Regensburg
Regensburg
Germany

Vanessa Falcao
Department of Biochemistry
University of Sao Paulo
Sao Paulo
Brazil

Emilio Fernández
Departamento de Bioquímica
y Biología Molecular
Campus de Rabanales
Universidad de Córdoba
Córdoba
Spain

Samuel P. Fletcher
Department of Cell Biology
The Skaggs Institute
for Chemical Biology
The Scripps Research Institute
La Jolla, California
U.S.A.

Markus Fuhrmann
Sloning BioTechnology
Puchheim
Germany

Aurora Galván
Departamento de Bioquímica
y Biología Molecular
Campus de Rabanales
Universidad de Córdoba
Córdoba
Spain

Maria L. Ghirardi
National Renewable
Energy Laboratory
Basic Science Center
Golden, Colorado
U.S.A.

David González-Ballester
Departamento de Bioquímica
y Biología Molecular
Campus de Rabanales
Universidad de Córdoba
Córdoba
Spain
and

Department of Plant Biology
The Carnegie Institution
of Washington
University of Stanford
Stanford, California
U.S.A.

Christoph Griesbeck
Kompetenzzentrum
für Fluoreszente Bioanalytik
Universität Regensburg
Regensburg
Germany

Arthur R. Grossman
Department of Plant Biology
The Carnegie Institution
Stanford, California
U.S.A.

Markus Heitzer
Kompetenzzentrum
für Fluoreszente Bioanalytik
Universität Regensburg
Regensburg
Germany

Peter Kroth
Fachbereich Biologie
Universität Konstanz
Konstanz
Germany

Rosa León
Departamento de Química
y Ciencia de Materiales
Facultad de Ciencias Experimentales
Universidad de Huelva
Huelva
Spain

Stephen P. Mayfield
Department of Cell Biology
The Skaggs Institute
for Chemical Biology
The Scripps Research Institute
La Jolla, California
U.S.A.

Anastasios Melis
Department of Plant
and Microbial Biology
University of California
Berkeley, California
U.S.A.

Machiko Muto
Department of Cell Biology
The Skaggs Institute
for Chemical Biology
The Scripps Research Institute
La Jolla, California
U.S.A.

Saul Purton
Algal Research Group
Department of Biology
University College London
London
U.K.

Sathish Rajamani
Biophysics Program
Ohio State University
Columbus, Ohio
U.S.A.

Participants

vii

Richard Sayre
Biophysics Program
and
Department
of Plant Cellular and Molecular
Biology
Ohio State University
Columbus, Ohio
U.S.A.

Michael Seibert
National Renewable
Energy Laboratory
Basic Science Center
Golden, Colorado
U.S.A.

Surasak Siripornadulsil
Department of Microbiology
Khon Kaen University
Khon Kaen
Thailand

Moacir Torres
Department of Biochemistry
University of Sao Paulo
Sao Paulo
Brazil

Agustín Vioque
Instituto de Bioquímica
Vegetal y Fotosíntesis
Universidad de Sevilla-CSIC
Sevilla
Spain

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

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 fixation¹ 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 reinhardtii*, which was first transformed in 1989 by complementation of *nit1* and *arg7* mutations with homologous nitrate reductase⁹ and argininosuccinate lyase genes,¹⁰ respectively. Since then a significant number of selectable markers, promoters, and new procedures

*Corresponding Author: Rosa León—Departamento de Química y Ciencia de Materiales, Facultad de Ciencias Experimentales, Avda, Fuerzas Armadas s/n, Universidad de Huelva, 21007-Huelva, Spain. Email: rleon@uhu.es

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

Division	Species	Method	Ref.
Dinoflagellates	<i>Amphidinium</i>	Silicon carbide whiskers	37
	<i>Symbiodinium</i>	Silicon carbide whiskers	37
Diatoms	<i>Phaeodactylum tricornutum</i>	Bombardment	36,32
	<i>Cyclotella cr�ptica</i>	Bombardment	34
	<i>Navicula saprophila</i>	Bombardment	34
	<i>Cylindrotheca fusiformis</i>	Bombardment	35
	<i>Thalassiosira weissflogii</i>	Bombardment	36
Chlorophyceae	<i>Chlamydomonas</i>	Glass beds	39
		Electroporation	42
		Silicon carbide whiskers	41
	<i>Chlorella ellipsoidea</i> *	Bombardment	10,44
		Agrobacterium	45
		Bombardment	19
		Electroporation	23
		Electroporation	20
		Protoplast transformation	21
		Bombardment	26
<i>Dunaliella salina</i>	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 H₂ (see Chapter 10), recombinant vaccines (Chapter 11) and bioremediation (Chapter 9) are some examples.