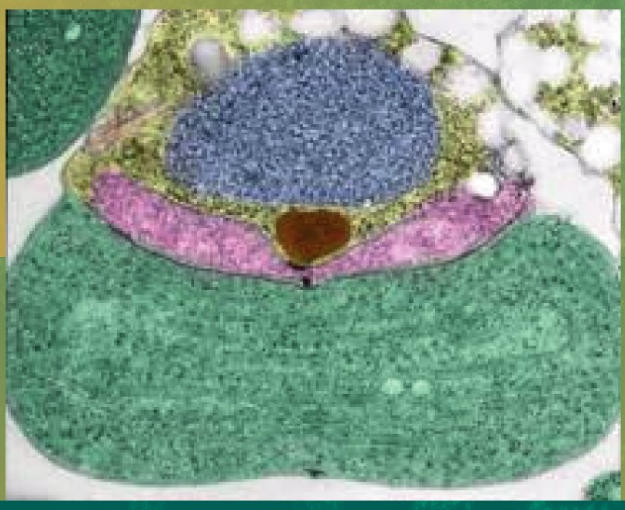
Cellular Origin, Life in Extreme Habitats and Astrobiology 13

Joseph Seckbach
David J. Chapman
Editors

Red Algae in the Genomic Age



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RED ALGAE IN THE GENOMIC AGE

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 13

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The Hebrew University of Jerusalem, Israel

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Red Algae in the Genomic Age

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Cover illustration: Top center: *Cyanidioschyzon merolae* (Cyanidioceae) cell. Artificial colors shows the chloroplast in green, mitochondrium in pink, peroxisome in dark brown, the nucleus in blue, and the Golgi Apparatus at the left of the nucleus colored light brown. Donated by **Dr. Shin Ya Miyagishima** (Research Unit in RIKEN, Japan).

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DEDICATION

We dedicate this volume to Professor Aharon Oren (The Hebrew University of Jerusalem) for all his contributions to the *Cellular Origin, Life in Extreme Habitats and Astrobiology* series, and his share for our understanding of algae over a wide spectrum of investigation.

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INTRODUCTION TO RED ALGAE IN THE GENOMIC AGE

Red algae (Rhodophyta) are mainly marine algae whose chlorophyll and carotenoids are masked by the red or purplish pigments, the phycobiliproteins, phycoerythrins and phycocyanins. Some groups of the Rhodophyta serve as a source of agar and carrageenan, used as an ingredient in food. *Porphyra*, is grown for production of Nori and Zicai (in Japan alone the total annual production of nori amounts to over billions of US dollars). Among other edible red seaweeds are dulse (*Palmaria*) and Irish moss (*Chondrus*).

The great majority of the Rhodophyta are macroscopic, multicellular, benthic marine algae, found in the intertidal and in the subtidal to depths of up to 40 m. and occasionally deeper. In addition there are some genera of single celled planktonic forms and some freshwater genera in addition to the thermophilic/acidophilic Cyanidiophyceae. The main Rhodophyta reserves are typically floridean starch, and floridoside while the unicellular Cyanidiophycea (see further) produce also glycogens. The walls of the red algae are made of cellulose, with the sulfated galactans agar, and carrageenans. The cell walls of the unicellular Cyanidiophyceae are mainly proteinaceous**.**

This volume is number thirteen in the series *Cellular Origins, Life in Extreme Habitats and Astrobiology* (COLE) see: www.springer.com/series/5775. The unicellular Rhodophyta are discussed from various aspects such as origins, evolution, ecology, habitats, and the genomic features. Among the unicellular rhodophytans are the thermo-acidophiles *Cyanidiophyceae* (*Cyanidium caldarium* group) which are discussed in detail in this book. Additional chapters deal with the current genomic aspects of some rhodophytes, such as *Porphyra* (*Bangiaceae*), *Cyanidioschyzon merolae,* and *Galdieria sulphuraria* (Cyanidiaceae), which have been recently sequenced.

In this volume we gathered contributors, authorities in their fields, from a dozen countries. It is hoped that this book will benefit a wide range of readers, from undergraduate students to professional scholars, in the fields of biology, microbiology, phycology, and ecology. We express our deep appreciation to the reviewers for their time and effort in evaluating and reviewing the chapters.

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Biodata of **Joseph Seckbach**, editor (with **David J. Chapman**) of this volume and the author of the chapter ***"Overview of the Cyanidia"***

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Among his publications are books, scientific articles in the lines of phytoferritin, cellular evolution, acidothermophilic algae, and life in extreme environments. He also edited and translated several popular books. Dr. Seckbach is the co-author (with R. Ikan) of the *Chemistry Lexicon* (1991, 1999) and a co-editor of *Proceeding of Endocytobiology VII Conference* (Freiburg, Germany, 1998) and the *Proceedings of Algae and Extreme Environments meeting* (Trebon, Czech Republic, 2000). His new edited volume (with Richard Gordon) entitled *Divine Action and Natural Selection: Science, Faith, and Evolution* has been published by World Scientific Publishing Company. His recent interest is in the field of enigmatic microorganisms and life in extreme environments.

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FOREWORD

Red Algae in Genome Age Book

Most people reading this book have childhood memories about being enthralled at the beach with those rare and mysterious living forms we knew as seaweeds. We were fascinated at that time by their range of red hues and textures, and most of all, their exotic beauty. To a scientist, red algae represent much more than apparent features. Their complex forms have attracted morphologists for centuries; their intricate life cycles have brought more than one surprise to plant biologists familiar only with ferns and flowering plants; their unusual tastes have been appreciated for millennia, and their valuable chemical constituents have been exploited for nearly as long, most recently by biotech companies; their diversity in marine, freshwater, and terrestrial environments has offered centuries of engaging entertainment for botanists eager to arrange them in orderly classification systems; still, the red algae continue to teach us how many more challenges need to be overcome in order to understand their biodiversity, biological functions, and evolutionary histories.

This book is about the genomics of red algae. The reader will not be dissatisfied to find that the rhodophytes provide a plethora of genomic surprises to keep us ever more interested in our never-ending biological pursuits. The red algae are one of the most ancient photosynthetic eukaryotes that, along with the green algae and glaucophytes, initiated long and rather complicated evolutionary pathways. Along the way, through further endosymbioses, they changed other life forms resulting in even more complex genomic lineages. Representatives of extant red algae found in our planet are the survivors of significant adventures. Their evolutionary histories may be understood if we decipher their stories. Genomics may provide the key to appreciating the problems these lineages have been confronting since their early appearance on our planet, and the elegant adaptive solutions that have insured their survival over millennia.

The editors of *Red Algae in Genome Age* have accomplished a remarkable task in bringing together a group of scientists with a wide range of expertise, including systematics, ecology, biotechnology, molecular biology and medicine, bioinformatics, extremophile biology, and evolutionary biology. The chapters in this volume have been organized into six parts preceded by an introductory section. The initial part corresponds to the Origin and Evolution of Red Algae (1) followed by General Studies of Rhodophyta (2). The majority of the chapters are assembled in parts (3) Genomic Studies and Biotechnology and (4) Cyanidia. Next follows (5) Biochemistry and Physiology, and a final part (6) comprising the Outlook and Summary.

Some books on the biology of red algae have had a profound affect on our understanding of these remarkable organisms. They have functioned as a conduit for learning at both undergraduate and graduate levels. These publications are also a mirror of the state of the art of the times, akin to milestones in the history of rhodophytan research. I fondly recall Kylin's monumental treatise published in 1956 on rhodophytan genera, *Die Gattungen der Rhodophyceen*; his ordinal morphology-based classification system endured for almost half a century. Seventeen years passed before the publication of Dixon's *Biology of the Rhodophyta* in 1973; this book introduced more dynamic features of the biology of red algae. Another seventeen-year gap preceded the 1990 publication of *Biology of Red Algae* edited by K. M. Cole and R. G. Sheath, which introduced the readers to a modern overview of rhodophytan biology, highlighting major advancements in molecular biology, DNA analysis, physiology, and genetics. Seventeen years later, J. Seckbach and D. Chapman began organizing the publication of this book, *Red Algae in Genome Age* – what a timely publication!

The preceding publication leaps have witnessed gigantic technological developments and remarkable algal discoveries that have re-invigorated our incessantly growing understanding of the tree of life. *Red Algae in Genome Age* will introduce phycologists and scientists in general to novel approaches to better understanding the rhodophtyes. Where terms such as gene expression, genome architecture, genome lineages, phylogenomics, to name just a few, are becoming an essential part of our research on the biology of red algae.

The red algae continue to fascinate us with their enigmatic beauty and well-kept secrets. Glimpses into future genomic rhodophytan research can be perceived from the stimulating chapters in this book. *Red Algae in Genome Age* represents a significant contribution to the field of algal genomics. It will be of great value to phycologists, scientists in general, university mentors and mentees, as well as to a wider audience interested in red algae and their uses.

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We thank all our contributors for taking part in this volume, *Red Algae in Genome Age.* We are grateful to out external peer reviewers for the time and effort they expended in helping ensure the quality of the contributions: C. Amsler, R. Castenholz, R.L. Chapman, D. Cheney, C. Deatombes, P. Geigenberger, B. Green, T. Hase, E. Gantt, J. Huisman, I. Korf, G. Kraemer, A.W. Larkum, J. Lopez-Bautista, C. Maggs, Ch. McKay, C. Oesterhelt, G. Saunders, M. Verlaque, A. Weber, and J. Zertuche-Gonzalez. If any reviewers' name have been omitted, we apologize for the oversight. We wish also to acknowledge all colleagues who assisted us with their good advice for this volume. Special appreciations are due to Dr. Shin-Ya Miyagishima (Riken, Japan) for preparing the photo for this book's cover. Last but not least the senior editor (JS) is grateful to his wife, Fern Seckbach, for her patience, understanding and assistance during the compiling of this book (and other volumes in the series).

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PART 1:
ORIGIN AND EVOLUTION

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THE CHLOROPLAST DIVISION MACHINERY: ORIGIN AND EVOLUTION

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1. Introduction

It is widely believed that chloroplasts and mitochondria arose from bacterial endosymbionts related to cyanobacteria and α-proteobacteria, respectively (reviewed in Reyes-Prieto et al., 2007; Fig. 1). Although most of their genes have either been lost or transferred to the host nuclear genome, they retain several features similar to bacteria. Both organelles contain nucleoids and ribosomes, and neither are synthesized de novo. Chloroplasts multiply by binary division, as do cyanobacteria (Leech et al., 1981; Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Kuroiwa, 1991; Kuroiwa et al., 1998). However, the chloroplast genome does not contain sufficient information for division, indicating that the host eukaryotic cell genome regulates the division of chloroplasts. Understanding the mechanism of chloroplast division should provide important insights into the question of how the eukaryotic host cell coordinates its own division with that of the endosymbiont to establish the permanent endosymbiotic relationship.

The first important insight into chloroplast division was obtained from studies of the red algae *Cyanidium caldarium* in 1986. A ring structure was identified on the cytosolic side of the outer envelope membrane at the chloroplast division site by electron microscopy (Mita et al., 1986). After the identification in *C. caldarium*, this structure, called a plastid-dividing (PD) ring, was found in a number of plant and algae species (summarized in Kuroiwa et al., 1998; Fig. 2). These findings suggested that chloroplast division is performed by a division apparatus, which forms at the division site in a manner similar to actomyosin contractile rings in cytokinesis. The history up to the discovery of the PD ring, including details on the structure, has been summarized elsewhere (Leech et al., 1981; Possingham and Lawrence, 1983; Boffey and Lloyd, 1988; Kuroiwa, 1991; Kuroiwa et al., 1998).

In the decade following the PD ring discovery, the molecular mechanisms underlying the division process began to be understood, in particular because of the identification of two self-assembling GTPase proteins. A homolog of bacterial division protein FtsZ was identified in the nuclear genome of *Arabidopsis thaliana* in 1995 (Osteryoung and Vierling, 1995). A member of the dynamin family of eukaryotic GTPases was identified in the red alga *Cyanidioschyzon merolae*

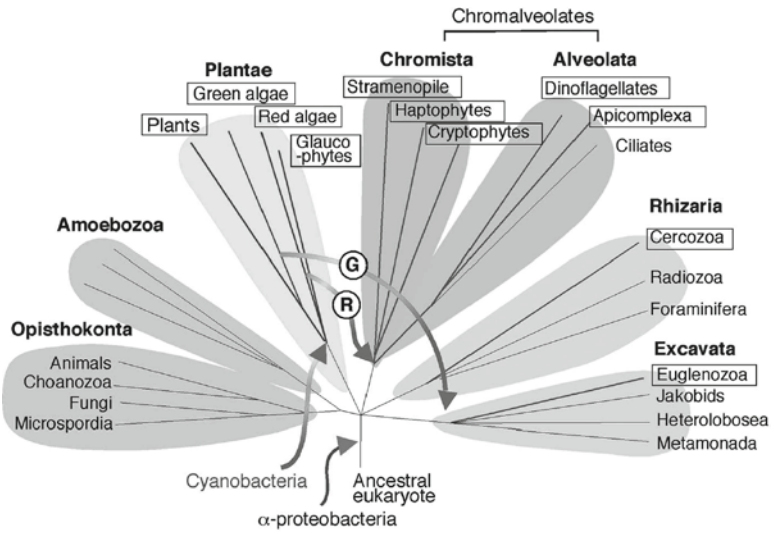

Figure 1. The phylogenetic relationship of eukaryotes and the origin of chloroplasts. The lineages indicated by squares have chloroplasts or nongreeen plastids. After the establishment of mitochondria, chloroplasts were established in the ancestor of Plantae by primary endosymbiosis and enslavement of an ancestral cyanobacterium. The ancestor of Plantae evolved to form glaucophytes, red algae, green algae, and land plants. A red algal cell that was engulfed and enslaved (secondary endosymbiosis) by the ancestral chromalveolate gave rise to chloroplasts in Chromista and Alveolata. Ciliates, such as *Paramecium* and *Tetrahymena*, and parasitic stramenopiles, such as oomycetes, lost the secondary chloroplasts. A green algal cell was enslaved by the ancestors of excavates (including Euglenozoa) and Rhizaria (including chlorarachniophytes). The dinoflagellates replaced their existing red algal secondary chloroplasts (shared with other alveolates) with another chloroplast by engulfing stramenopiles, haptophytes, or green algae. Details are summarized in Reyes-Prieto et al. (2007). G and R on the arrows indicate chloroplasts of green algal and red algal origin, respectively.

(Miyagishima et al., 2003a) and *A. thaliana* (Gao et al., 2003). Both FtsZ and dynamin were shown to localize at the chloroplast division site and to be involved in the division process (reviewed in Osteryoung and Nunnari, 2003; Miyagishima et al., 2003b; Fig. 2). These results revealed that a part of the division machinery, including FtsZ, is descended from the cyanobacterial endosymbiont, whereas the other part, which includes dynamin, is evolved from the eukaryotic host cell.

The mechanism of chloroplast division has been extensively examined in the red algae *C. merolae* and the land plant *A. thaliana*. Studies using *C. merolae* have yielded important information, in part owing to the highly synchronous system of organellar division (Suzuki et al., 1994). In addition, the organellar and nuclear genomes were completely sequenced (Matsuzaki et al., 2004; Misumi et al., 2005; Nozaki et al., 2007) and the results showed that the genomes have very simple contents. Studies in *C. merolae* and *A. thaliana* have shown commonalities in the division mechanisms of red algae and land plants. However, certain factors identified in *A. thaliana* do not exist in other lineages of photosynthetic eukaryotes,

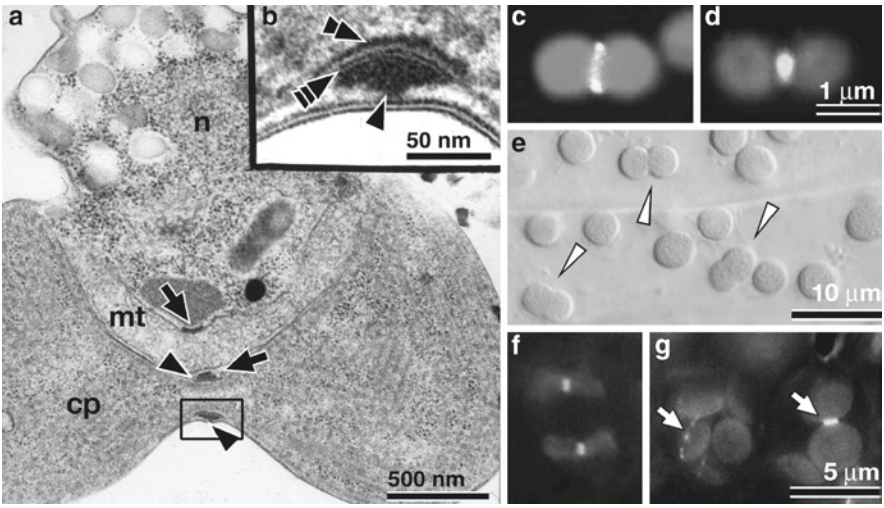

Figure 2. The PD (plastid-dividing), FtsZ, and dynamin rings. (**a**) Electron micrographs of a *C. merolae* cell containing a dividing chloroplast and mitochondrion. *cp,* chloroplast; *mt,* mitochondrion; *n,* nucleus. (**b**) Magnified cross-section of the PD ring, corresponding to the boxed-region in **a**. The PD ring is composed of an outer ring (on the cytosolic side of the outer envelope; *arrowheads*), a middle ring (in the intermembrane space; *triple arrowheads*), and an inner ring (on the stromal side of the inner envelope; *double arrowheads*). The MD (mitochondrion-dividing) ring, a structure similar to the PD ring, is also observed at mitochondrial division site (*arrows*). (**c, d**) Immunofluorescence images of the FtsZ (CmFtsZ2; **c**) and dynamin (CmDnm2; **d**) rings during *C. merolae* chloroplast division. The bright fluorescence indicates the localization of each protein and the transparent fluorescence is chloroplast autofluorescence. (**e**) Dividing chloroplasts in petiole cells of *A. thaliana*. The dividing chloroplasts are indicated by *arrowheads*. (**f, g**) FtsZ2-1-GFP (**f**) and GFP-ARC5 dynamin (**g**) localize at the chloroplast division sites in *A. thaliana.* Note that dynamin localizes at the division site as a discontinuous ring (*the right arrow*) and then forms a continuous ring during a late stage of the division site constriction (*the left arrow*).

suggesting that the division machinery has been modified differently in different lineages since the system was established in the ancestral algae. To summarize the results from several lineages of eukaryotes, we first describe a brief summary of the current understanding of the origin and evolution of chloroplasts.

2. The Evolution and Spread of Chloroplasts in Eukaryotes

Chloroplasts are found in several eukaryotic lineages in addition to red algae, green algae, and plants. For example, remnants of the chloroplast (called "apicoplasts") are found in apicomplexan parasites that cause serious human diseases, including malaria and toxoplasmosis (reviewed in Reyes-Prieto et al., 2007; Fig. 1). Given the diversity of these lineages, the mechanisms of chloroplast division might be expected to vary. However, since current understanding in evolutionary biology

holds that all chloroplasts have the same endosymbiotic origin (a cyanobacterium), it is reasonable to postulate that the mechanism of chloroplast division was, in principle, first established in algal chloroplasts. As described in this chapter, the overall mechanism of chloroplast division is conserved across diverse species, although several modifications have been introduced. The results obtained from different species must be understood in the context of the evolutionary history of chloroplasts.

Evolutionary studies have suggested that three types of endosymbiosis (primary, secondary, and tertiary) have resulted in the establishment of chloroplasts in eukaryotes (Reyes-Prieto et al., 2007; Fig. 1). In primary endosymbiosis, which occurred in the ancestor of Plantae including glaucophytes, red algae, and Chlorophyta (land plants and green algae), engulfment of a prokaryotic cyanobacterium gave rise to a chloroplast bound by two membranes. In secondary endosymbiosis, previously nonphotosynthetic eukaryotes engulfed eukaryotic algae (either red or green algae), which were then reduced to secondary chloroplasts. Secondary chloroplasts have three or four envelope membranes; the third and fourth membranes (going from the innermost to outermost) are thought to correspond to the plasma membrane of the engulfed alga and the phagosomal membrane of the host cell, respectively (Reyes-Prieto et al., 2007).

The most favored hypothesis is that secondary endosymbiosis of a red alga may have resulted in the origin of the chromalveolates, including cryptophytes, haptophytes, stramenopiles, and apicomplexa (Cavalier-Smith, 1999; Fast et al., 2001). Plastids (apicoplasts) of apicomplexan parasites are not capable of photosynthesis.

According to the above endosymbiotic theory, the division of the first and second envelope membranes of secondary chloroplasts and their contents is the same phenomenon as seen in primary chloroplast division. In this chapter, we first describe chloroplast division in Plantae, especially focusing on the red algae *C. merolae* and the land plant *A. thaliana*, and then expand the description to the secondary chloroplasts.

3. The Mechanism of Chloroplast Division in Model Organisms

3.1. MECHANISMS DERIVED FROM THE CYANOBACTERIAL ENDOSYMBIONT

3.1.1. Involvement of FtsZ Proteins in Chloroplast Division

The mechanism of chloroplast division began to be revealed at the molecular level with the identification of FtsZ proteins encoded in plant and algal nuclear genomes. FtsZ is a bacterial division protein that was identified by the analyses of filamentous, temperature-sensitive (*fts*) *Escherichia coli* mutants (reviewed in Weiss, 2004; Harry et al., 2006). Owing to defects in cytokinesis, *fts* mutants elongate to form filaments. FtsZ, one of the several Fts proteins, is a GTPase structurally similar to tubulin (Lowe and Amos, 1998) and is conserved in most bacteria and

archaea. It self-assembles into a ring structure beneath the cytoplasmic membrane at the division site. The formation of the FtsZ ring is the first known event at the division site, and it initiates the recruitment of the other proteins that comprise the bacterial division complex (Weiss, 2004; Harry et al., 2006). Therefore, FtsZ is thought to play a central role in prokaryotic cell division.

A gene encoding chloroplast-targeted FtsZ was found in the *A. thaliana* nuclear genome in 1995 (Osteryoung and Vierling, 1995). The amino acid sequence of the protein is most similar to cyanobacterial FtsZ, supporting an endosymbiotic origin for FtsZ (Osteryoung and Vierling, 1995). After the identification in *A. thaliana*, *ftsZ* genes were found in several additional species of plants and algae.

Gene disruption experiments in the moss *Physcomitrella patens* (Strepp et al., 1998) and the expression of antisense RNA in *A. thaliana* (Osteryoung et al., 1998) inhibited chloroplast division generating giant chloroplasts. These results indicated that FtsZ proteins are required for chloroplast division in plants. Subsequently, immunocytochemical studies showed that plant FtsZ proteins form ring structures at chloroplast division sites in land plants (Mori et al., 2001; Vitha et al., 2001; Fig. 2) and the red algae *C. merolae* (Miyagishima et al., 2001c; Fig. 2). The FtsZ proteins have an N-terminal transit peptide and localize on the stromal side of the inner envelope membrane (Mori et al., 2001; Miyagishima et al., 2001c; McAndrew et al., 2001; Kuroiwa et al., 2002). These results revealed that at least a part of the chloroplast division machinery is descended from cyanobacteria, although the genes have been transferred to the host nuclear genome.

3.1.2. Other Chloroplast Division Proteins of Cyanobacterial Origin

The suggestion that the FtsZ descended from the cyanobacterial endosymbiont is involved in chloroplast division led to the identification and characterization of additional homologs of bacterial division proteins in plants and algae. The molecular mechanism of bacterial cell division has been extensively studied in *Escherichia coli*, a Gram-negative γ-proteobacterium, and *Bacillus subtilis*, a Gram-positive bacterium (Weiss, 2004; Harry et al., 2006). In *E. coli*, once an FtsZ ring is formed at the division site, the remaining division proteins, ZipA, FtsA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsW, FtsI, and FtsN, are recruited to the site in this order to activate septation (Weiss, 2004; Harry et al., 2006). Proteins regulating FtsZ ring formation in bacteria have also been identified. These proteins include MinC, MinD, MinE, and DivIVA, which regulate the placement of the FtsZ ring (Weiss, 2004; Harry et al., 2006). In *E. coli*, MinC, MinD, and MinE are involved in the positioning of FtsZ ring, whereas *B. subtilis* does not have MinE, and DivIVA, MinC, and MinD are involved in the process.

Of the several division genes identified in *E. coli* and *B. subtilis*, *ftsE, ftsI, ftsQ, ftsW, ftsZ, minC, minD, minE, sepF* (*ylmF*), and *divIVA* are well conserved in cyanobacterial genomes (Miyagishima et al., 2005), and some of these proteins have been confirmed to be involved in cyanobacterial cell division as well (Mazouni et al., 2004; Miyagishima et al., 2005). Studies in cyanobacteria also have identified additional genes specific to cyanobacteria that are involved in cell

division, including *ftn2* (Koksharova and Wolk, 2002) and certain other genes (Koksharova and Wolk, 2002; Miyagishima et al., 2005).

Searches of the *A. thaliana* nuclear genome identified genes homologous to cyanobacterial *minD* (Colletti et al., 2000) and *minE* (Itoh et al., 2001). The results of antisense RNA, overexpression, or gene disruption experiments confirmed that these genes are involved in chloroplast division (Colletti et al., 2000; Itoh et al., 2001; Reddy et al., 2002; Maple et al., 2002).

In addition to the reverse genetic studies on *ftsZ*, *minD*, and *minE*, forward genetic studies in *A. thaliana* yielded the identification of other chloroplast division genes of cyanobacterial origin. *A. thaliana* mutants with altered numbers of chloroplasts per mesophyll cell were collectively named *arc* (accumulation and replication of chloroplasts) mutants (Pyke and Leech, 1992, 1994; Pyke, 1999; Marrison et al., 1999). Cells in most of the *arc* mutants contain very large chloroplasts and the numbers of chloroplasts per cell are reduced, suggesting that the chloroplasts can grow but their division is blocked in these mutants. The *arc6* mutation was mapped to an *A. thaliana* nuclear gene that is orthologous to cyanobacterial *ftn2* (Vitha et al., 2003). The ARC6 protein was shown to localize at the chloroplast division site, spanning the inner envelope membrane (Vitha et al., 2003), and later Ftn2 was shown to localize at the cell division site in cyanobacteria (Mazouni et al., 2004).

These results indicate that certain cyanobacterial cell division genes in addition to *ftsZ* were transferred to the nuclear genome of the eukaryotic host, where they play roles in chloroplast division. However, database searches revealed that *ftsE, ftsI, ftsQ, ftsW,* and *divIVA,* which are present in cyanobacterial genomes, are missing from the *A. thaliana* genome (Miyagishima et al., 2005). The genome of *C. merolae* has only the *ftsZ* gene from among the above-mentioned cyanobacterial division genes (Miyagishima et al., 2005). Examination of both the *A. thaliana* and *C. merolae* genomes indicate that the majority of bacterial cell division genes were lost after endosymbiosis.

3.1.3. Possible Division Factors of Cyanobacterial Origin

Three genes in addition to the ones described earlier have also been implicated in chloroplast division in *A. thaliana*. When either the *ARTEMIS* (Fulgosi et al., 2002) or *CRUMPLED LEAF* (Asano et al., 2004) gene is disrupted, chloroplast division is impaired, with giant chloroplasts generated in the cells. Similarly, knock-down or overexpression of the *AtSulA*/*GC1* gene reportedly impairs chloroplast division (Maple et al., 2004; Raynaud et al., 2004). These three genes are most likely descended from the cyanobacterial endosymbiont (Fulgosi et al., 2002; Asano et al., 2004; Maple et al., 2004; Raynaud et al., 2004). ARTEMIS contains a YidC/Oxa1/Alb3 translocase-like domain and localizes at the inner envelope membrane of chloroplasts (Fulgosi et al., 2002), and the CRUMPLED LEAF protein localizes on the outer envelope membrane (Asano et al., 2004). Neither the ARTEMIS nor the CRUMPLED LEAF protein localizes specifically to the division sites, and their roles in chloroplast division are unknown.

AtSulA/GC1 is annotated as a protein similar to *E. coli* SulA, which is induced by the SOS response, and inhibits FtsZ polymerization, delaying cell division until DNA damage is repaired in *E. coli* (Weiss, 2004; Harry et al., 2006). However, the similarity of primary structures between AtSulA/GC1 and SulA is not significant. At present, it is not known whether AtSulA/GC1 is directly involved in chloroplast division.

Factors derived from the bacterial peptidoglycan synthesis pathway have also been suggested to play a role in chloroplast division. Some antibiotics that inhibit bacterial peptidoglycan synthesis also inhibit chloroplast division in the moss *P. patens* (Kasten and Reski, 1997; Katayama et al., 2003), and some genes in *A. thaliana* and *P. patens* encode the homologs of enzymes that act in peptidoglycan synthesis in bacteria (Katayama et al., 2003). Disruption of the genes in *P. patens* impaired chloroplast division (Machida et al., 2006). However, peptidoglycans have never been detected in chloroplasts (except for glaucophyte chloroplasts), and plant genomes do not have the full complement of genes that are required for peptidoglycan synthesis in bacteria. In chloroplasts, homologs of the bacterial peptidoglycan synthesis pathway may have other functions.

3.2. MECHANISMS DERIVED FROM THE EUKARYOTIC HOST

3.2.1. Paralogous Evolution of FtsZ Proteins

Most bacteria (including cyanobacteria) have only one *ftsZ* gene, whereas chloroplast-containing eukaryotes have two types of phylogenetically distinct *ftsZ* genes of cyanobacterial origin, suggesting that *ftsZ* gene duplication occurred subsequent to the endosymbiotic event. Because these genes (named *FtsZ1* and *FtsZ2*, Osteryoung et al., 1998) are present both in land plants and in green algae (Wang et al., 2003; Stokes and Osteryoung, 2003), *ftsZ* gene duplication appears to have occurred before plants branched from green algae. In *A. thaliana*, FtsZ1 and FtsZ2 colocalize at the stromal side of the inner envelope membrane (McAndrew et al., 2001; Kuroiwa et al., 2002), even when the expression level and assembly pattern of each was altered experimentally (McAndrew et al., 2001). Like chlorophytes, red algae (Miyagishima et al., 2004) and stramenopiles (Kiefel et al., 2004; Miyagishima et al., 2004) also have two types of *ftsZ* genes, but phylogenetic analyses indicate that gene duplication arose independently in chlorophytes, red algae, and stramenopiles (Miyagishima et al., 2004).

In both chlorophytes and nonchlorophytes, one of the two FtsZ proteins contains a short, conserved domain at the C-terminus (C-terminal core domain) that is also conserved in bacterial FtsZ (Osteryoung and McAndrew, 2001; Kiefel et al., 2004; Miyagishima et al., 2004), whereas the other FtsZ protein does not have the C-terminal core domain. In bacteria, the C-terminal core domain of FtsZ binds to FtsA and ZipA (Weiss, 2004; Harry et al., 2006). Although neither FtsA nor ZipA has been found in eukaryotic genomes, it was shown that ARC6 specifically interacts with the core domain of FtsZ2, but not with FtsZ1, which

lacks the core domain (Maple et al., 2005). Although it is still not known how FtsZ proteins lacking the C-terminal domain contribute to chloroplast division, depletion of either *ftsZ* gene in *A. thaliana* inhibits chloroplast division, suggesting that both FtsZ1 and FtsZ2 are required (Osteryoung et al., 1998).

Recently, a third FtsZ-like protein was found in *A. thaliana* by cloning of the *ARC3* gene. The *arc3* mutant cells have giant chloroplasts like other *arc* mutants, suggesting that chloroplast division is inhibited in the mutant (Pyke and Leech, 1992, 1994). *ARC3* encodes a protein that has an FtsZ-like N-terminal region and a C-terminal region homologous to a region of phosphatidylinositol-4-phosphate 5-kinase (Shimada et al., 2004). The ARC3 protein localizes on the stromal side of the chloroplast division site (Maple et al., 2007), like other FtsZ proteins. Functional studies in *A. thaliana* suggest that ARC3 is involved in the placement of the FtsZ ring in chloroplasts (Maple et al., 2007).

3.2.2. The PD Ring

The plastid-dividing (PD) ring was first identified as an electron-dense structure encircling the chloroplast division site in the red algae *C. caldarium* (Mita et al., 1986). After that, similar structures have been identified in green algae, land plants, and stramenopiles (summarized in Kuroiwa et al., 1998). In most cases, the PD rings were detected as a double ring structure, with one ring (the outer PD ring) (Mita et al., 1986) on the cytosolic face of the outer envelope and one ring (the inner PD ring) (Hashimoto, 1986) on the stromal face of the inner envelope. In the red algae *C. merolae*, a middle PD ring was also identified in the intermembrane space (Miyagishima et al., 1998a; Fig. 2).

The ultrastructures and behaviors of the two (or three) PD rings were extensively characterized in the red algae *C. caldarium* (Mita et al., 1986) and *C. merolae* (Miyagishima et al., 1998b, 1999, 2001a). The studies showed that the structure and behavior of the three rings are different, suggesting that each ring has distinct function and is composed of distinct sets of proteins.

Characterization of the PD rings in green algae (Chida and Ueda, 1991; Ogawa et al., 1995) and the land plant *Pelargonium zonale* (Kuroiwa et al., 2002) provided results similar to those in the red algae, implying that the rings are composed of orthologous proteins in several lineages. Electron-dense structures were also reported in dividing chloroplasts of the glaucophyte *Cyanophora paradoxa* (Iino and Hashimoto, 2003). In *C. paradoxa*, the ring structure was observed at the stromal side of the inner envelope membrane and no structure was observed on the cytosolic side of the outer envelope membrane at the division site. At present, whether or not this ring structure corresponds to the inner PD ring is unknown.

Although FtsZ was once believed to be a component of the inner PD ring (Kuroiwa et al., 1998), or both the inner and outer PD rings (Osteryoung et al., 1998), the FtsZ ring was ultimately shown to be a structure that is distinct and separable from the PD rings (Miyagishima et al., 2001c). The FtsZ ring is

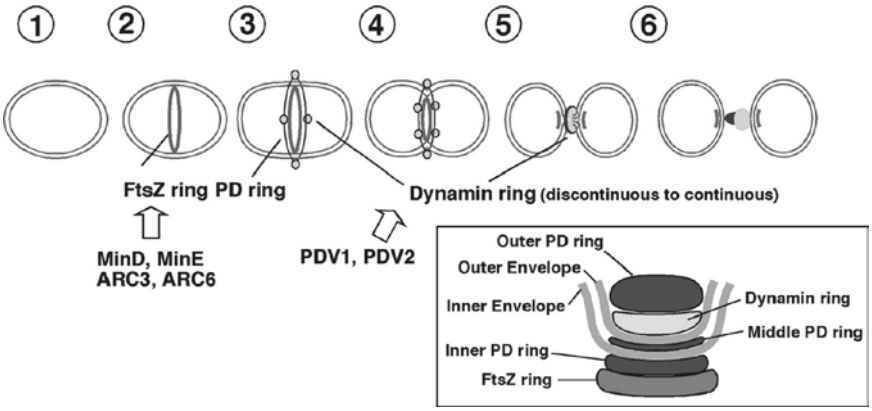

Figure 3. Sequence of events in chloroplast division. (1) Chloroplasts elongate. (2) An FtsZ ring forms at the division site. (3) The inner PD ring forms, followed by the formation of middle and the outer PD rings (the inner and the middle PD rings are not shown.). Constriction of the division site commences once the PD ring is assembled. (4) During constriction, cytosolic dynamin patches are recruited to the cytosolic side of the division site to form a discontinuous ring. (5) A continuous dynamin ring forms in the late stage of division. The FtsZ ring disassembles, followed by disassembly of the inner and middle PD rings. (6) When division is complete, the remnant of the outer PD ring, which is now located between the two daughter chloroplasts, disassembles. In contrast, the remnant of the dynamin ring is retained by one of the two daughter organelles and subsequently disappears. Details are described in Miyagishima et al. (2001a, c, 2003a).

positioned on the interior side of the inner PD ring, and it assembles prior to the PD rings (Miyagishima et al., 2001c; Kuroiwa et al., 2002; Fig. 3).

Although none of the PD ring components have yet been identified, the outer PD ring forms as a bundle of filaments (Kuroiwa et al., 1998). Biochemical dissection in *C. merolae* has shown that the outer PD ring is a bundle of 5-nm filaments (Miyagishima et al., 2001b; Yoshida et al., 2006). These filaments are believed to be novel structures, based on their dimensions and their biochemical stability (Miyagishima et al., 2001b). No electron-dense structures such as the PD ring have been observed at the division sites of bacteria, including cyanobacteria. Thus, the inner and outer PD rings (and the middle PD ring of *C. merolae*) do not appear to correspond to any parts of the bacterial division machinery, suggesting that they arose in the eukaryotic host after endosymbiosis.

3.2.3. Dynamin-Related Proteins

The above results from the study of the PD rings raise the possibility that some of the components of chloroplast division machinery are derived from the eukaryotic host. A mixed origin for the chloroplast division machinery was demonstrated by the identification of a dynamin-related protein in chloroplast division.

The dynamin and dynamin-related proteins are a family of large GTPase proteins involved in fission and fusion in several eukaryotic membrane systems

(reviewed in Praefcke and McMahon, 2004). Dynamin, the first member of the dynamin family to be identified, assembles into a ring at the neck of clathrin-coated pits, where it appears to play a role in pinching off vesicles from the plasma membrane (Takei et al., 1995; Hinshaw and Schmid, 1995). Several dynamin homologs have since been identified, and some of these homologs have been shown to be involved in mitochondrial division (reviewed in Praefcke and McMahon, 2004). Analogous to the function of conventional dynamin at the plasma membrane, DRP-1 (Labrousse et al., 1999) and its yeast ortholog Dnm1p (Bleazard et al., 1999; Sesaki and Jensen, 1999) are found on the cytosolic side of the mitochondrial division sites.

Because genes homologous to *ftsZ* have not been found in animals as well as fungi, it has been suggested that, in mitochondria, dynamin has replaced the function of FtsZ (Erickson, 2000; Margolin, 2000). However, both mitochondrial FtsZ (descended from α-proteobacteria) and dynamin (CmDnm1) were identified in the red algae *C. merolae* and were shown to localize at the mitochondrial division site (Nishida et al., 2003). The study showed that the FtsZ ring forms in mitochondria before the onset of the division site constriction, whereas dynamin localizes at the division site only during the late stage of constriction (Nishida et al., 2003). These results suggested that the functions of FtsZ and dynamin are distinct.

Analyses of the complete organellar and nuclear genome sequences of *C. merolae* revealed only two dynamin-related genes, *Cmdnm1* and *Cmdnm2* (Miyagishima et al., 2003a; Matsuzaki et al., 2004). Phylogenetic study revealed that CmDnm2 and its orthologs are specific to plant and algae, and it was shown that CmDnm2 localizes at the cytosolic side of the chloroplast division site (Miyagishima et al., 2003a; Fig. 2). Simultaneously, the *arc5* mutation of *A. thaliana* was mapped to the plant-specific dynamin-related protein (Gao et al., 2003). *arc5* mutant chloroplasts are enlarged owing to the division defect, but frequently they are dumbbell-shaped (Pyke and Leech, 1994; Robertson et al., 1996; Marrison et al., 1999) and associated with the PD ring (Robertson et al., 1996).

Dynamin localizes at the chloroplast division site as a discontinuous ring during the earlier phase of constriction and the continuous dynamin ring forms during the late stage of constriction both in *C. merolae* (Miyagishima et al., 2003a) and *A. thaliana* (Gao et al., 2003; Miyagishima et al., 2006; Fig. 2), unlike the FtsZ and PD rings. The phenotype and localization data indicate that the dynamin ring is distinct from the outer PD ring. The phenotype of the *arc5* mutant suggests that dynamin is required only in the later stage of division site constriction or pinching-off of the membrane. However, an *in vitro* study using chloroplast division apparatus (complex of the FtsZ, PD, and dynamin rings) isolated from the red algae *C. merolae* suggests that dynamin is required both for constriction and fission (Yoshida et al., 2006). At present, it is not known how dynamin is biochemically involved in chloroplast division.

3.2.4. PDV1 and PDV2

PDV1 and PDV2 were identified in *A. thaliana* as proteins functionally related to dynamin. Both the *pdv1* and *pdv2* mutants have enlarged chloroplasts with

constrictions, as does the *arc5* mutant (Miyagishima et al., 2006). PDV1 and PDV2 are outer-envelope spanning proteins paralogous to each other. PDV1 was shown to localize at the chloroplast division site and the localization is discontinuous, as in the case of ARC5 dynamin. Both PDV1 and PDV2 are required for the recruitment of ARC5 dynamin protein to the division site. PDV1 and PDV2 have a putative coiled-coil domain, which is exposed to the cytosolic side of the outer envelope membrane, implying that they interact with each other or with other proteins, including dynamin (Miyagishima et al., 2006). At present, however, it is not known whether PDV1 or PDV2 biochemically interact with dynamin, and if so how they recruit dynamin to the division site.

4. Organization and Evolution of Chloroplast Division Machinery

4.1. ORGANIZATION AND DYNAMICS OF THE DIVISION MACHINERY

Over the past decade, several proteins were identified as components of the chloroplast division machinery. Detailed observation using the red algae *C. merolae* and molecular genetic study with *A. thaliana* have uncovered a part of the spatiotemporal relationship among the components (Marrison et al., 1999; Kuroiwa et al., 2002; Miyagishima et al., 2003a; Maple et al., 2005; Yoshida et al., 2006; summarized in Glynn et al., 2007; Maple and Møller, 2007), but studies on the specific details are still in progress.

Of the several components of the chloroplast division machinery, the FtsZ, PD, and dynamin rings are well conserved across the lineages studied (for the detail, see below). The FtsZ, PD, and dynamin rings form in this order at the division site (Miyagishima et al., 2003a; Fig. 3). The FtsZ ring forms first, inside the chloroplast (Fig. 3, stage 2), and then the PD ring forms from the inner to the outer rings before division site constriction (Fig. 3, stage 3). During constriction of the division site, a patch-like structure including dynamin is recruited to the cytosolic surface of the division site (Fig. 3, stages 3 and 4). During the late constriction stage, the continuous dynamin ring forms on the cytosolic side of the outer envelope membrane (Fig. 3, stage 5). In addition, during this stage when constriction is still in progress, the FtsZ ring disassembles and FtsZ is excluded from the division site (Miyagishima et al., 2001c). The middle and inner PD rings disappear just before the completion of division, whereas remnants of the outer PD ring remain between the daughter chloroplasts, and remnants of the dynamin ring remain clinging to each daughter chloroplast (Fig. 3, stage 6). After completion of division, the remnants of the PD and dynamin rings dissolve in the cytosol (Miyagishima et al., 2001a, c, 2003a).

A very recent study using the chloroplast division apparatus isolated from *C. merolae* has advanced the understanding of the division complex. The study isolated the intact complex including the FtsZ, PD, and dynamin rings, and the result suggest that the rings were connected by unknown factors across the two

envelope membranes. Furthermore, it was shown that the isolated complex (rings) can constrict, most likely by the function of the dynamin protein (Yoshida et al., 2006). Further study using the isolated complex should yield critical insights into how the complex is organized and how the constrictive force is generated.

Studies using *A. thaliana* suggest that MinD and MinE regulate the positioning of the FtsZ ring formation as in bacteria. ARC3 is also suggested to be involved in this process. PDV1 and PDV2 recruit ARC5 dynamin to the division site. ARC6 is suggested to be involved in the formation/stabilization of the FtsZ ring (summarized in Glynn et al., 2007; Maple and Møller, 2007). However, none of these proteins (MinD, MinE, ARC3, PDV1, and PDV2) are encoded in the *C. merolae* genome (for the detail, see below).

4.2. CONSERVATION OF AND DIFFERENCES IN THE DIVISION MACHINERY AMONG SEVERAL DIFFERENT LINEAGES

The chloroplast division machinery consisting of the FtsZ, PD, and dynamin rings must have been established before branching took place between red and green algae, because the same mechanism is observed both in the red algae *C. merole* and the land plant *A. thaliana.* These three rings of the red algae have probably been passed on to secondary chloroplasts based on the following results.

FtsZ sequences have been identified in glaucophytes (Sato et al., 2007) and in species that have chloroplasts of red algal secondary endosymbiotic origin (stramenopiles, and a cryptomonad; Fraunholz et al., 1998; Kiefel et al., 2004; Miyagishima et al., 2004). Phylogenetic studies showed that the stramenopile and cryptomonad FtsZ is derived from engulfed red algae (Miyagishima et al., 2004). The cryptomonad *ftsZ* gene is present in its nucleomorph (residual nucleus of the engulfed red alga) (Fraunholz et al., 1998), whereas all other *ftsZ* genes are found in host nuclear genomes.

The outer and inner PD rings have also been observed in stramenopiles *Heterosigma akashiwo* (Hashimoto, 1997) and *Mallomonas* species (Beech and Gilson, 2000; Weatherill et al., 2007). In these species, which have secondary chloroplasts of a red algal origin, the inner and the outer PD rings were observed at the first and second (from inside out) envelope membranes of the four envelope membranes, consistent with the hypothesis that the inner pair of membranes is derived from engulfed red algal chloroplasts.

Probable orthologs of the chloroplast division dynamin are encoded in genomes of the stramenopiles *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Table 1). These dynamin sequences are most closely related to the chloroplast division dynamin of red algae. These facts suggest that the dynamin gene was transferred from the nuclear genome of the engulfed red algae into the nuclear genome of the ancestral chromalveolates.

Given the theory that the chloroplasts of chromists and alveolates have the same red algal origin (Reyes-Prieto et al., 2007), the ancestor of the chromalveolates

once had the FtsZ, PD, and dynamin rings for chloroplast division. Consistent with this prediction, the PD ring-like structure was observed in the apicomlexa *Toxoplasma gondii* (Matsuzaki et al., 2001). However, no *ftsZ* genes have been identified in apicomplexan genomes. Although apicomplexan species have dynamin-like genes, these genes are not closely related to the chloroplast division related dynamin proteins (Table 1), suggesting that *ftsZ* and the chloroplast division dynamin genes have been lost in apicomplexans. Although it is not presently known how the fission of apicoplasts occurs, mitotic spindles are required (Striepen et al., 2000).

Comparison between cyanobacterial, algal, and plant genomes suggest that most of the cyanobacterial division proteins were lost after endosymbiosis (Table 1). In addition, the particular cyanobacteria-derived division components, which are present in different eukaryotic lineages, vary (Table 1). In *A. thaliana*, homologs of *minD*, *minE*, *ftn6* (*ARC6*), and *ftsZ* are found in the nuclear genome, and no cyanobacterial cell division genes are found in the chloroplast genome (Table 1). In contrast, the chloroplast genomes of some green algae encode *minD, minE, ftsI*, and *ftsW* (summarized in Miyagishima et al., 2005). The nuclear and chloroplast genomes of the red alga *C. merolae* lack cyanobacterial division genes other than *ftsZ* (Matsuzaki et al., 2004; Misumi et al., 2005; Miyagishima et al., 2005; Table 1), and the nuclear genome of the stramenopile *T. pseudonana* possesses *ftsZ, minC,*

Table 1. Distribution of known chloroplast division genes.

	Division proteins descended from cyanobacteria					
Species	**ftsZ**	**ARC3**	**minC**	**minD**	**minE**	**ARC6**
Cyanidioschyzon merolae (Rhodophyta)	+	−	−	−	−	−
Thalassiosira pseudonana (Stramenopiles)	+	−	+	+	−	−
Phaeodactylum tricornutum (Stramenopiles)	+	−	−	−	−	−
Plasmodium falciparum (Alveolata)	−	−	−	−	−	−
Ostreococcus tauri (Chlorophyta)	+	−	−	+	+	+
Chlamydomonas reinhardtii (Chlorophyta)	+	−	−	+	+	+
Arabidopsis thaliana (Plants)	+	+	−	+	+	+

	Eukaryotic division proteins	
Species	**ARC5**	**PDV1/PDV2**
Cyanidioschyzon merolae (Rhodophyta)	+	−
Thalassiosira pseudonana (Stramenopiles)	+	−
Phaeodactylum tricornutum (Stramenopiles)	+	−
Plasmodium falciparum (Alveolata)	−	−
Ostreococcus tauri (Chlorophyta)	+	−
Chlamydomonas reinhardtii (Chlorophyta)	+	−
Arabidopsis thaliana (Plants)	+	+

+, identified; −, not identified. Both the nuclear and chloroplast genomes were searched by BLAST programs. Of the known cyanobacterial cell division genes, *ftsE, ftsI, ftsQ, ftsW, ylmF* (*sepF*), *divIVA,* and *ftn6* were not identified in the listed genomes.

and *minD* (Table 1). The differences in gene contents other than *ftsZ* indicate that cyanobacterial division genes were lost in different ways in different lineages.

The FtsZ-like protein ARC3 is involved in chloroplast division in *A. thaliana*, as described earlier (Pyke and Leech, 1992, 1994; Marrison et al., 1999; Shimada et al., 2004; Maple et al., 2007). However, no *ARC3* homologs are encoded in the genomes of the moss *P. patens*, green algae, or red algae (Table 1), suggesting that the gene emerged only in land plants, after the moss had branched out. Similarly, BLAST searches did not reveal proteins similar to PDV1 and PDV2 in species other than land plants (Table 1).

4.3. SIMILARITY BETWEEN CHLOROPLAST AND MITOCHONDRIAL DIVISION MACHINERY

The most striking suggestion given by recent studies is that almost the same event occurred during the establishment of mitochondria. Some of the protists have retained α-proteobacterial FtsZ for mitochondrial division (Beech et al., 2000; Beech and Gilson, 2000; Gilson et al., 2003; Kiefel et al., 2004; Nishida et al., 2003; Takahara et al., 2000, 2001). The MD (mitochondrion-dividing) ring, which has a structure similar to the PD ring, has been found in red algae (Kuroiwa et al., 1993, 1995, 1998; Fig. 2), the true slime mold *Physarum polycephalum* (Kuroiwa et al., 1977, 2006) and the stramenopile *Nannochloropsis oculata* (Hashimoto, 2004). A dynamin-related protein is also involved in mitochondrial division in diverse eukaryotic species (Praefcke and McMahon, 2004). During mitochondrial division in the red algae *C. merolae*, the FtsZ, MD, and dynamin rings form in this order, the same as in chloroplast division (Nishida et al., 2003). These facts suggest that mitochondria carried out division by almost the same mechanisms as chloroplasts soon after they were established in ancestral eukaryotes. These results suggest that the host cell used the same strategy to regulate the division of the cyanobacterial endosymbiont as it did for the α-proteobacterial endosymbiont.

5. Summary

Like its ancestor, chloroplasts use bacterial division system based on the FtsZ ring and certain associated factors, all of which are now encoded in the host nuclear genome. The majority of the cyanobacterial division factors other than *ftsZ* have been lost differently in the different lineages. In their place, several new factors have been added by the eukaryotic host. For example, the *ftsZ* gene has been duplicated and modified, PD rings were most likely added by the eukaryotic host, and a member of the dynamin family of proteins evolved to regulate chloroplast division. The principal features of the chloroplast division system based on the FtsZ, PD, and dynamin rings were transmitted to the ancestor of chromalveolates by a red algal secondary endosymbiosis. The chloroplast division machinery may

reflect primeval features of mitochondrial division, because similar but paralogous components are involved in mitochondrial division in protists.

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EVOLUTIONARY HISTORY AND TAXONOMY OF RED ALGAE

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1. Evolutionary Relationship of Red Algae

The red algae (Rhodophyta) form a distinct photosynthetic eukaryotic lineage that consists of around 6,000 species including unicellular to large multicellular taxa (http://www.algaebase.org/). The red algae are unique among eukaryotes in lacking both flagella and centrioles during their entire life cycle (Gabrielson et al., 1990; Graham and Wilcox, 2000). Pit connections, pit plugs, and a triphasic life cycle that are mostly found in the Florideophyceae are also distinguishing characters of the red algae. The photosynthetic organelle (plastid) of red algae is bounded by two membranes and contains chlorophyll-*a*, phycocyanin, and phycoerythrin as photosynthetic pigments. These pigment complexes, organized in phycobilisomes, are located on the surface of unstacked thylakoid membranes to capture light energy. As a storage product, the red algae produce granulated floridean starch in the cytoplasm that is different from green algal starch. In addition to these unique features, the monophyly of red algae is strongly supported by nuclear, plastid, and mitochondrial gene trees (Freshwater et al., 1994; Ragan et al., 1994; Van de Peer and De Wachter, 1997; Burger et al., 1999; Yoon et al., 2002b, 2004).

1.1. ORIGIN OF THE RED ALGAL PLASTID AND ITS RELATIONSHIP TO OTHER PLANTAE

It is generally agreed that the red algal plastid originated via primary endosymbiosis. Under this scenario, a photosynthetic cyanobacterium was captured by a heterotrophic protist and the prokaryote was eventually transformed into a plastid. This protistan chimera was an evolutionary success story and gave rise to the three major photosynthetic lineages, i.e., the red, green (including land plants), and glaucophyte algae (Bhattacharya and Medlin, 1995; Delwiche et al., 1995; McFadden, 1999; Bhattacharya et al., 2004; Rodriguez-Ezpeleta et al., 2005;

Hackett et al., 2007a). The kingdom Plantae was established to include these three primary plastid-containing groups that all contain a double-membrane bound photosynthetic organelle that lies free in the cytosol (Cavalier-Smith, 1998). Plantae monophyly is generally recovered when inferring trees with plastid genes (Yoon et al., 2002b, 2004), nuclear genes that encode plastid targeted proteins (Li et al., 2006; Nosenko et al., 2006), and some nuclear gene data (Moreira et al., 2000; Rodriguez-Ezpeleta et al., 2005 [for counter-arguments to Plantae monophyly, see Stiller et al., 2001; Nozaki et al., 2003; Stiller and Harrell, 2005]). The strongest support for Plantae monophyly comes from a 143-gene phylogeny that had however limited taxon sampling (Rodriguez-Ezpeleta et al., 2005), and a 16-gene tree with 46 taxa that included all of the eukaryotic supergroups (Hackett et al., 2007a).

In addition to phylogenetic evidence, Plantae monophyly is supported by shared components of the plastid machinery. For example, the evolutionary history of plastid targeted metabolite translocators in red and green algae (including land plants) suggests a common ancestry of these taxa. These data show that a single gene recruitment event occurred in the ancestral alga followed by duplications and diversification of gene functions that are unique to red and green algae (Weber et al., 2006). These steps were critical for plastid establishment because the plastid translocators connect the biochemistry of the host and organelle to facilitate a regulated flow of metabolites. A similar piece of evidence is provided by the finding of over 55 genes of chlamydial origin in Plantae, many of which are shared by red, green, and glaucophyte algae (Huang and Gogarten, 2007; Moustafa et al., 2008). These genes were recruited from environmental Chlamydiae early in Plantae evolution and have been mantained by algae and plants over the ensuing 1 billion years because of the key functions they provide to plastid and other host-derived functions. The set of plastid targeted translocators and Chlamydiae genes shared uniquely by red and green algae (final assesssment of the glaucophyte component awaits completion of the nuclear genome sequence of *Cyanophora paradoxa*; D. Bhattacharya and J. Boore, unpublished data) provides overwhelming evidence that these taxa shared a single common ancestor in which these genetic innovations were established. Finally, Plantae also contain a unique, shared protein import system that is embedded in their organelle membranes (i.e., TOC-TIC translocons, [McFadden and van Dooren, 2004; Reumann et al., 2005]) that also supports a single origin of these taxa. Given that Plantae monophyly is now all but proven, a final piece of data that was recently published demonstrates that within this supergroup, the glaucophytes are the earliest diverging algal lineage. Reyes-Prieto and Bhattacharya (2007) used a concatenated data set of 19 conserved nuclear-encoded plastid targeted proteins to provide robust phylogenetic support for the "glaucophytes first" hypothesis. This result clarifies a long-standing issue in Plantae evolution that was not adequately resolved using plastid gene/genome data and nuclear genes of nonplastid function (e.g., Nozaki et al., 2003; Rodriguez-Ezpeleta et al., 2005; Yoon et al., 2005).

1.2. RED ALGAL FOSSILS AND LINEAGE DIVERGENCE TIMES

There are only a handful of reported unicelluar microfossils. Because of their simple morphology and absence of distinguishable characters to suggest a specific affinity, microfossils are generally recognized as cyanobacteria, although some may be of eukaryotic derivation. For example, the unicelluar fossils with a mucilage sheath from the 1,900-million-year-old (Ma) Gunflint Formation (Ontario, Canada), *Eosphaera tyleri* are likely to be red algae (Barghoorn and Tyler, 1965; Tappan, 1976); however, this suggestion is still hotly debated. Branched microfossils, acritarchs, were reported from the 1,450 Ma Roper Group, Australia (Javaux et al., 2001). Acritarchs may be fungi, red algae, or of unknown affinity; however, it is generally agreed that these fossils were most likely to have been marine eukaryotic algae (Butterfield, 2005; Porter, 2006).

Besides these controversal microfossils, there are two taxonomically unambiguous remains that may provide a key timeline for red algal evolution. *Bangiomorpha pubescens* is a multicellular filamentous fossil that was found in the 1,200 Ma Hunting Formation from Somerset Island, Canada (Butterfield, 2000). These fossils are almost completely preserved and regarded as the oldest taxonomically resolved eukaryotic fossil (Fig. 1). *B. pubescens* is very similar to modern *Bangia* species in cell division pattern, holdfast shape, and gross morphology. In addition to multicellularity, the fossils have spores/gametes that putatively represent differentiation due to sexual reproduction. Therefore, these fossils may indicate the occurrence of a major red algal radiation in the Mesoproterozoic. The other clear examples of red algal fossils are florideophycean algae (*Thallophyca* and *Paramecia*) from the ca. 600 Ma Doushantuo Formation in China (Xiao et al., 1998, 2004). The Doushantuo pseudoparenchymatous thalli are likely to be extinct coralline algae that contain carposporophytes, suggesting the presence of a triphasic life cycle that is typical for extant Florideophyceae. Because the coralline algae are not an early diverging lineage in the Florideophyceae (Saunders and Kraft, 1997), the diversification of Florideophyceae (e.g., Hildenbrandiophycidae is the earliest diverging florideophycean lineage) must have occurred prior to the age of Doushantuo (600 Ma).

On the basis of six reliable fossil data points including *Bangiomorpha* and the Doushantuo algae, Yoon et al. (2004) estimated an evolutionary timeline for photosynthetic eukaryotes using a six-gene data set and "relaxed" clock molecular phylogenetic methods. These authors suggested ca. 1,500 Ma for divergence of the red and green algal lineages, 1,370 Ma for the Cyanidiophyceae split, ca. 1,300 Ma for the secondary endosymbiosis that eventually gave rise to the plastid in chromalveolate lineages, and ca. 800 Ma for the divergence of the Florideophyceae from the Bangiales. They postulated that the noncyanidiophyceaen lineages (i.e., five major unresolved red algal groups) radiated around 1,200 Ma over a relatively short evolutionary time period (Yoon et al., 2004). A widely cited molecular clock analysis that contradicts a Mesoproterozoic origin of red algae was done by Douzery et al. (2004) who suggested that Plantae originated between 892 and 1,162 Ma, which is about 500 million years later than the estimate made by Yoon et al. (2004)

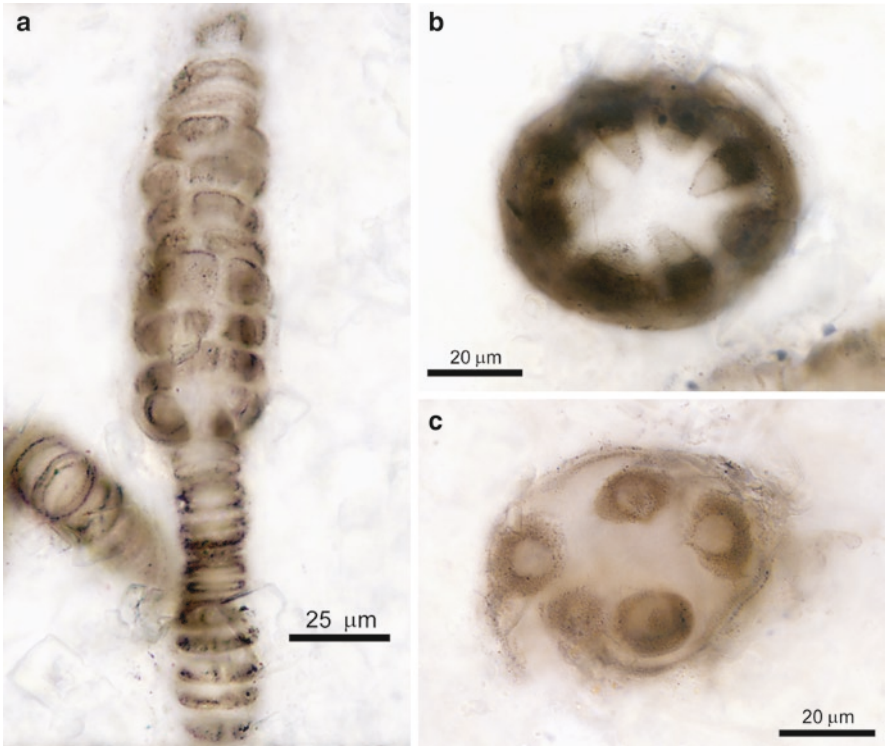

Figure 1. *Bangiomorpha pubescens* fossils from the ca. 1,200 Ma Hunting Formation, Somerset Island, arctic Canada. (**a**) Mature thallus showing both uniseriate and multiseriate portions of a filament; (**b**) transverse cross-section of a multiseriate filament showing eight radially arranged wedge-shaped cells, exactly comparable with the longitudinal intercalary division in extant *Bangia*; (**c**) transverse cross-section of a four-part multiseriate filament, which has differentiated to form spheroidal spores that is comparable with the asexual monospores of extant *Bangia* (courtesy of N. J. Butterfield).

and a subsequent analysis by Hackett et al. (2007b). It should be noted that the Douzery et al. (2004) date is derived from a large 129-protein data set, but with limited taxon sampling. If true, this work is clearly inconsistent with the broadly accepted fossil data cited above by proposing a 928 Ma divergence for the red algae and 872 Ma split for stramenopiles. The reasons for these inconsistencies remain unclear and await future in-depth analyses that incorporate genome data from a broader diversity of eukaryotes.

2. Classification of the Rhodophyta

A classification system for a group of organisms should reflect its phylogeny. A reliable phylogeny ideally uses both broad taxon sampling and sufficient phylogenetic information (Yoon et al., 2006).

The Rhodophyta is one of the three phyla in the kingdom Plantae. Red algae have traditionally been classified into two classes Bangiophyceae and Florideophyceae (Gabrielson et al., 1985), or two subclasses Bangiophycidae and Florideophycidae of the class Rhodophyceae (Dixon, 1973). This morphology-based taxonomy was challenged with the onset of numerical cladistics and molecular systematics. Garbary and colleagues suggested paraphyly of the Bangiophyceae *sensu lato* based on cladistic analysis of morphological characters (Gabrielson et al., 1985; Gabrielson et al., 1990; Garbary and Gabrielson, 1990). Molecular systematic studies (Freshwater et al., 1994; Ragan et al., 1994; Saunders and Kraft, 1997; Oliveira and Bhattacharya, 2000; Müller et al., 2001) using nuclear or plastid genes (16S SSU, 18S SSU, and *rbc*L) confirmed that Bangiophyceae *sensu lato* is not a monophyletic clade and reported that the Porphyridiales are paraphyletic, including at least three independent lineages (Porphyridiales-1, -2, and -3). The monophyly of the Bangiales and the Florideophyceae was also proposed using these data (e.g., Oliveira and Bhattacharya, 2000; Müller et al., 2001). Recent multigene phylogenetic analyses (albeit with a narrow sampling of taxa) found that the Cyanidiophyceae is the earliest diverging red algal lineage (Yoon et al., 2002b, 2004). In summary, both single and multigene approaches, with or without a broad taxon sampling, identify the major lineages within the red algae but fail to resolve their interrelationships.

Saunders and Hommersand (2004) revised the current classification system and proposed a new taxonomic scheme. In addition to the phylum Rhodophyta, they suggested the new phylum Cyanidiophyta with a single class Cyanidiophyceae under the new subkingdom Rhodoplantae. A three-subphylum (Rhodellophytina, Metarhodophytina, and Eurhodophytina) with four-class (Rhodellophyceae, Compsopogonophyceae, Bangiophyceae, and Florideophyceae) system was proposed for the phylum Rhodophyta. It is noteworthy that they attempted to incorporate red algal molecular phylogenies and ultrastructural characters (i.e., Golgi–ER association) into their taxonomic system that together provided a useful definition of classes in the red algae. They also established the subphylum Eurhodophytina to define the strongly supported monophyletic group of class Bangiophyceae and Florideophyceae. However, they defined the paraphyletic class Rhodellophyceae *classis nova* that includes three independent lineages (i.e., Porphyridiales, Stylonematales, and Porphyridiales-1, *sensu* Müller et al., 2001).

Yoon et al. (2006) conducted a comprehensive molecular systematic analysis using a broad taxon sampling with multigene analyses (Table 1). They included most of the phylogenetic lineages of Bangiophyceae *sensu lato* (25 genera, 48 taxa) to represent red algal diversity. On the basis of the finding of seven well-supported lineages, they proposed a new classification system of the Rhodophyta that contains two new subphyla, the Cyanidiophytina with a single class, the Cyanidiophyceae, and the Rhodophytina with six classes, the Bangiophyceae, Compsopogonophyceae, Florideophyceae, Porphyridiophyceae *classis nova*, Rhodellophyceae, and Stylonematophyceae *classis nova.* They also described a new order, Rhodellales, and a new family, Rhodellaceae. Although they used

Table 1. Current taxonomic system of the red algae according to Yoon et al. (2006).

Kingdom Plantae Haeckel
Phylum Rhodophyta Wettstein
Subphylum Cyanidiophytina Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Cyanidiophyceae Merola, Castaldo, De Luca, Gambardella, Musacchio et Taddei
Order Cyanidiales Christensen
Subphylum Rhodophytina Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Bangiophyceae Wettstein
Order Bangiales Nägeli
Class Compsopogonophyceae Saunders et Hommersand
Order Compsopogonales Schmitz in Engler et Prantl
Order Erythropeltidales Garbary, Hansen, et Scagel
Order Rhodochaetales Bessey
Class Florideophyceae Cronquist
Subclass Hildenbrandiophycidae Saunders et Hommersand
Order Hildenbrandiales
Subclass Nemaliophycidae Christensen
Order Acrochaetiales, Balbianiales, Balliales, Batrachospermales, Colaconematales, Corallinales, Nemaliales, Palmariales, Rhodogorgonales, Thoreales
Subclass Ahnfeltiophycidae Saunders et Hommersand
Order Ahnfeltiales
Subclass Rhodymeniophycidae Saunders et Hommersand
Order Bonnemaisoniales, Ceramiales, Gelidiales, Gigartinales, Gracilariales, Halymeniales, Nemastomatales, Plocamiales, Rhodymeniales
Class Porphyridiophyceae Yoon, Müller, Sheath, Ott, et Bhattacharya
Order Porphyridiales Kylin ex Skuja
Class Rhodellophyceae Cavalier-Smith
Order Rhodellales Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Stylonematophyceae Yoon,Müller, Sheath, Ott, et Bhattacharya
Order Stylonematales Drew

multigene data sets (i.e., seven-genes 2,564 amino acid sequences, nine-genes 10,463 bp), the multigene phylogenies still failed to resolve interrelationships between the noncyanidiophycean lineages (Fig. 2). This may not primarily reflect insufficient phylogenetic information, but rather a rapid radiation of the red algal lineages over a relatively short evolutionary timeframe. Therefore, the seven-class system may be a reasonable classification for the red algae.

3. Seven Classes of Rhodophyta

3.1. CLASS CYANIDIOPHYCEAE

The Cyanidiophyceae is an asexual, unicellular red algal class that belongs to the subphylum Cyanidiophytina. These taxa thrive in acidic and high temperature conditions around hot springs or acidic sulfur fumes (Pinto et al., 2003).

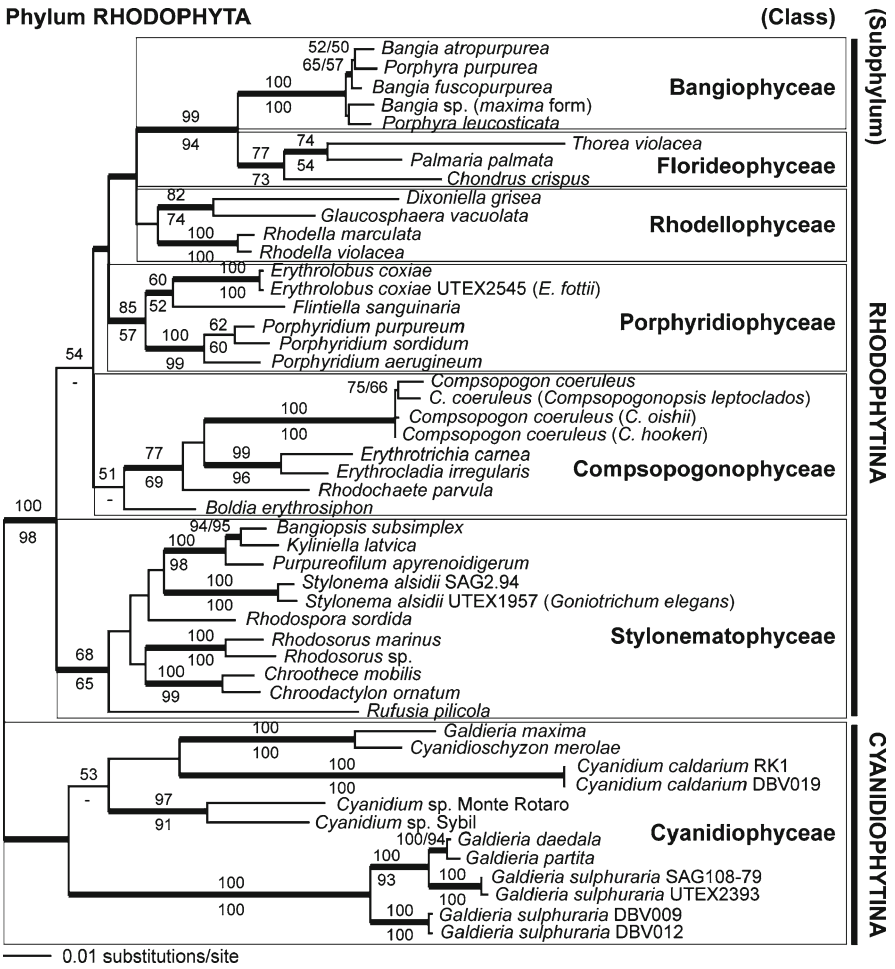

Figure 2. Maximum likelihood (ML) phylogeny of the red algae using the combined plastid protein sequences of *psa*A and *rbc*L. The results of a ML bootstrap analysis are shown above the branches, whereas the values below the branches result from a maximum parsimony bootstrap analysis. The thick branches indicate clades to the right with a greater-than-95% Bayesian posterior probability.

The Golgi apparatus in these species associates with the ER. The Cyanidiophyceae is a well-supported monophyletic group that diverged earliest within red algae around 1,370 Ma prior to the chromalveolate red algal secondary endosymbiosis (i.e., according to Yoon et al., 2004). Traditionally, the Cyanidiophyceae was classified into one order Cyanidiales, two families Cyanidiaceae and Galdieriaceae, and three genera *Cyanidium* (1 sp.), *Cyanidioschyzon* (1 sp.), and *Galdieria* (5 spp.). Because of their simple morphology, only seven species are recognized to date (Merola et al., 1981; Sentsova, 1991; Ott and Seckbach, 1994; Albertano et al., 2000; Pinto et al., 2003, 2007). It is, however, now clear that the diversity of the

Cyanidiophyceae was underestimated. Ciniglia et al. (2004) used an environmental PCR survey to find the hidden biodiversity among Cyanidiophyceae and suggested six putative family-level taxa within the cyanidiophycean lineage; the *Galdieria* lineage (as *Galdieria*-A), the endolithic *Galdieria* (*Galdieria*-B), the mesophilic *Cyanidium* lineage, the *Cyanidium* lineage, the *Cyanidioschyzon* lineage, and the *G. maxima* lineage (Fig. 2), which is essentially consistent with other molecular phylogenetic studies (Gross et al. 2001; Yoon et al., 2002a, 2006; Pinto et al., 2003). For example, the endolithic *Galdieria*-B was recently established as the new species *Galdieria phlegrea* by Pinto et al. (2007). On the basis of molecular phylogenetic analyses (Ciniglia et al., 2004), they conducted additional eco-physiological studies to characterize this new species. *G. maxima* and *Cyanidioschyzon merolae* showed a close sister group relationship; however, *G. maxima* is very different from *C. merolae* with regard to many morphological features (e.g., spherical vs. ellipsoidal cell shape, thick cell wall vs. lack of cell wall, facultative heterotroph vs. obligate autotroph, endospore vs. binary fission). It is clear that the current taxonomic scheme does not reflect the underlying phylogenetic relationships. Therefore, there needs to be a thorough revision of the classification system of the Cyanidiophyceae at the higher level (i.e., family level) as well as a comprehensive sampling of potentially new taxa (e.g., environmental PCR survey) to characterize the hidden biodiversity in this group.

3.2. CLASS BANGIOPHYCEAE

Bangiophyceae is a group of red algae that has simple unbranched filaments or a leaf-shaped thallus. The monophyly of the Bangiophyceae is supported by most molecular phylogenetic studies (Oliveira and Bhattacharya, 2000; Müller et al., 2001; Broom et al., 2004; Yoon et al., 2006). The Bangiophyceae grows in both marine and freshwater habitats. A biphasic life cycle is common in the group that includes macroscopic gametophyte and microscopic conchocelis phases. The monophyly of theBangiophyceae and Florideophyceae is supported by morphological as well as molecular data (see below). *Bangiomorpha* and *Porphyra* fossils are found in deposits of ages 1,200 Ma and 600 Ma, respectively (see above, Butterfield et al., 1988; Xiao et al., 1998; Butterfield, 2000). The Bangiophyceae includes one order Bangiales, one family Bangiaceae, and six genera *Bangia, Bangiadulcis, Dione, Minerva, Porphyra*, and *Pseudobangia*. Around 130 species are reported from the six genera, but the majority of species belong to *Bangia* (10 sp.) and *Porphyra* (117 sp.; http://www.algaebase.org/). Broom et al. (2004) collected 123 samples of *Bangia*, which had been recognized as ten species and reported significant hidden genetic diversity within this genus. Continuing efforts have erected the monotypic genera *Bangiadulcis, Dione, Minerva,* and *Pseudobangia* (Müller et al., 2005; Nelson et al., 2005; Nelson, 2007). However, it is obvious that a comprehensive taxonomic study is required for this group as well to uncover their "real" diversity.

3.3. CLASS FLORIDEOPHYCEAE

The monophyly of the Bangiophyceae and Florideophyceae is supported not only by molecular analyses but also by features of the reproductive cells, the association of the Golgi apparatus with ER/mitochondria, the presence of pit connections, and the presence of group I introns (Gabrielson et al., 1985, 1990; Freshwater et al., 1994; Ragan et al., 1994; Oliveira and Bhattacharya, 2000; Müller et al., 2001; Yoon et al., 2002b, 2004, 2006). Regarding, the close relationship between Bangiophyceae and Florideophyceae, Saunders and Hommersand (2004) suggested the subphylum Eurhodophytina to include these taxa. The class Floridiophyceae appears to have diverged from an ancestor of bangiophycean and florideophycean alga around 800 Ma (Yoon et al., 2004) and is the most taxonomically diverse of all red algal groups, including around 5,800 species. The Florideophyceae is one of the most successful algal groups in marine environments, perhaps because of their specialized life cycles (e.g., triphasic life cycle; gametophyte, carposporophyte, and tetrasporophyte phases). These red algae possess unique and distinctive female reproductive structures that are often used as taxonomic markers with respect to postzygotic development. On the basis of morphological and molecular phylogenetic data (Pueschel and Cole, 1982; Maggs and Pueschel, 1989; Harper and Saunders, 2002; Huisman et al., 2003; Saunders et al., 2004), the Florideophyceae is classified into four subclasses Hildenbrandiophycidae, Nemaliophycidae, Ahnfeltiophycidae, and Rhodymeniophycidae. Although internal relationships within these groups are still unclear, the four subclass system provides an overall view of florideophycean classification. For a detailed review of the Florideophyceae, see Saunders and Hommersand (2004).

3.4 CLASS COMPSOPOGONOPHYCEAE

The Compsopogonophyceae consists of one freshwater order Compsopogonales with two families Boldiaceae and Compsopogonaceae, and two marine orders Erythropeltidales and Rhodochaetales. Fourteen genera have been reported in this class (*Compsopogon, Boldia, Chlidophyllon*, *Erythrocladia, Erythropeltis, Erythrotrichia, Membranella, Porphyropsis, Porphyrostromium, Pulvinaster, Pyrophyllon, Rhodochaete, Sahlingia, Smithora*). All of these species are multicellular; *Erythrotrichia* is filamentous, *Smithora* and *Chlidophyllon* are parenchymatous blades, and *Boldia* is a single-cell-thick tubular form that is 20–75 cm in length. The monophyly of this group is supported by nuclear SSU rDNA and a plastid multigene analysis that show a sister relationship of Erythropeltidales and Rhodochaetales (Rintoul et al., 1999; Zuccarello et al., 2000; Müller, et al., 2001; Yoon et al., 2002b, 2006). This recently established class (Saunders and Hommersand, 2004) is characterized by having a Golgi–ER association, and floridoside as the low-molecular-weight carbohydrates (LMWC; Broadwater and Scott, 1994; Karsten et al., 2003). This class is also the only group outside the Bangiophyceae

and Florideophyceae in which sex (a complete life cycle or putative sexual structures) has been well documented (Magne, 1960, 1990) or proposed (Hawkes, 1988; Nelson et al., 2003). The divergence of this class parallel to the Bangiophyceae/Florideophyceae clade indicates that sex was established in the Rhodophyta before the putative fossil evidence provided by *Bangiomorpha*.

3.5. CLASS PORPHYRIDIOPHYCEAE

The Porphyridiophyceae includes one order Porphyridiales, one family Porphyridiaceae, and three unicellular genera, *Erythrolobus*, *Flintiella*, and *Porphyridium*. This unicellular red algal group contains a single branched or stellate plastid with the presense of a group II intron (genic position 1219) in the plastid-encoded *psa*A gene from *Flintiella* (Table 2). It was reported that *Flintiella* and *Porphyridium* have a Golgi association with ER/mitochondria (Scott et al., 1992) and possess floridoside as a LMWC (Karsten et al., 2003).

Table 2. The seven classes and all recognized genera of the Rhodophyta (excluding Florideophyceae) and some of their diagnostic characters (ER = endoplasmic reticulum; ER/M = ER-mitochondrion association; Nu = nucleus).

Class	Genus	Golgi association	Low-molecular-weight carbohydrate (LMWC)	Other characters
Cyanidiophyceae	*Cyanidium*	ER		
	Cyanidioschyzon			
	Galdieria	ER		
Bangiophyceae	*Bangia*		Floridoside, isofloridoside	
	Bangiadulcis			
	Dione			
	Minerva			
	Porphyra		Floridoside, isofloridoside	
	Pseudobangia			
Florideophyceae	Hundreds of genera			
Compsopogonophyceae	*Compsopogon*	ER	Floridoside	
	Boldia	ER	Floridoside	
	Chlidophyllon			
	Erythrocladia		Floridoside	
	Erythropeltis			
	Erythrotrichia		Floridoside	
	Membranella			
	Porphyropsis		Floridoside	
	Porphyrostromium			
	Pulvinaster	ER	Floridoside	

(continued)

Table 2. (continued)

Class	Genus	Golgi association	Low-molecular-weight carbohydrate (LMWC)	Other characters
	Pyrophyllon			
	Rhodochaete		Floridoside, digeneaside	
	Sahlingia		Floridoside	
	Smithora		Floridoside, isofloridoside	
Porphyridiophyceae	*Porphyridium*	ER/M	Floridoside	
	Erythrolobus			
	Flintiella	ER/M	Floridoside	Intron (1,219/psaA)
Rhodellophyceae	*Rhodella*	ER	Mannitol	Intron (91/psaA)
	Dixoniella	Nu	Mannitol	
	Glaucosphaera	Nu	Mannitol	
Stylonematophyceae	*Stylonema*	ER	Sorbitol, digeneaside	intron (229/psaA)
	Bangiopsis	ER	Sorbitol, digeneaside	Intron (229/psaA)
	Chroodactylon		Sorbitol	Intron (229/psaA)
	Chroothece			Intron (229/psaA)
	Colacodictyon			
	Empselium			
	Goniotrichopsis			
	Kylinella			
	Neevea			
	Purpureofilum	ER	Sorbitol, digeneaside	Intron (229/psaA)
	Rhodaphanes	ER	Sorbitol, digeneaside, trehalose	
	Rhodosorus		Sorbitol, digeneaside	Intron (229/psaA)
	Rhodospora		Sorbitol, dulcitol	Intron (229/psaA)
	Rufusia		Floridoside	Intron (229/psaA)

3.6. CLASS RHODELLOPHYCEAE

The class Rhodellophyceae was proposed by Cavalier-Smith (1998) and modified/retained by Saunders and Hommersand (2004); however, both definitions were not natural. Yoon et al. (2006) have revised the class as a group that includes unicellular red algae, *Dixoniella grisea, Glaucosphaera vacuolata*, *Rhodella violacea,* and *R. maculata.* The monophyly of the Rhodellophyceae is supported by multigene (Yoon et al., 2006) and 18S rDNA trees (Müller et al., 2001). This group contains mannitol as a LMWC (Karsten et al., 2003). Within the lineage, *D. grisea* and *G. vacuolata* are separated from two *Rhodella* species. Ultrastructural characters

support the relationship between *Dixoniella* and *Glaucosphaera*, which have a Golgi–Nuclear association with the presence of peripheral thylakoids, whereas *Rhodella* has a Golgi–ER association with the absence of peripheral thylakoids (Scott et al., 1992). Furthermore, group II introns were found at genic position 91 in the *psa*A gene only in two *Rhodella* species (*R. violacea*, *R. maculata*). Therefore, it may be possible to classify the two groups into separate orders or families. Additional work may show that more genera or families need to be established to encompass this diversity.

3.7. CLASS STYLONEMATOPHYCEAE

The Stylonematophyceae was proposed by Yoon et al. (2006) based on multigene phylogenetic analyses. This class includes pseudofilamentous or unicelluar taxa with thick mucilaginous walls and cells lacking pit plugs. With the single order Stylonematales, the single family Stylonemataceae contains 14 genera (*Bangiopsis, Chroodactylon, Chroothece, Colacodictyon, Empselium, Goniotrichopsis, Kylinella, Neevea, Purpureofilum, Rhodaphanes, Rhodosorus, Rhodospora*, *Rufusia,* and *Stylonema*) that have been reported mostly from marine habitats. Most genera have cells with a single stellate plastid with a pyrenoid. A Golgi–ER association with digeneaside and sorbitol as a LMWCs are diagnostic characters for this group (Broadwater and Scott, 1994; Karsten et al., 2003), although digeneaside is missing in *Chroodactylon* and dulcitol is present in *Rhodospora*. A group II intron was found at genic position 229 in the *psa*A gene from most of the species (Table 2). Recently, West and colleagues isolated two new genera *Rhodaphanes* and *Purpureofilum* belonging to this class (West et al., 2005, 2007). Most of the pseudofilamentous species in this group are found as epiphytes, and it is expected that new taxa will continue to be discovered.

4. Summary

The red algae are one of the most distinct eukaryotic groups. This photosynthetic lineage acquired its plastid from a cyanobacterium through primary endosymbiosis ca. 1,600 Ma, and then became a plastid donor to the chromalveolates through secondary endosymbiosis. This latter lineage is the most morphologically and taxonomically diverse eukaryotic group known. Therefore, the red algae have contributed significantly to eukaryote evolution. The classification of the Rhodophyta in a two-class system (i.e., Bangiophyceae and Florideophyceae) has been accepted by most red algal taxonomists for several decades. This morphological character-based system was, however, challenged by recent molecular systematic studies. It has been suggested that the Bangiophyceae *sensu lato* are paraphyletic and the Bangiales and Florideophyceae are monophyletic. Saunders and Hommersand (2004) suggested a two-phylum and five-class system for the red algae; however, it retains the paraphyletic class Rhodellophyceae. In contrast, Yoon et al. (2006)

conducted a molecular systematic analysis using multigene data with a broad taxon sampling and suggested two-subphyla and seven-class system that reflects phylogenetic relationships.

In this review, we hope that the reader appreciates that there remains significant hidden biodiversity among red algae. Novel taxa are being added to the red algae (e.g., Cyanidiophyceae, Bangiophyceae) and will significantly increase the number of species. This growth in biodiversity needs of course to be reflected in future taxonomic schemes. We also suggest that single gene approaches may not be sufficient to resolve interrelationships among higher-level groups of red algae. Multigene or genomic methods with a broad taxon sampling strategy (i.e., including all genera from at least the non-Florideophyceae) should allow us to resolve interrelationships between classes that will be a guide to establish a natural classification system for the red algae.

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PART 2:
GENERAL STUDIES
OF RHODOPHYTA

Hoffman
Dubinsky
Israel
Mitsunobu
West
Kumano
Shigeru

Biodata of **Razy Hoffman** and **Zvy Dubinsky**, authors of ***"Invasive and Alien Rhodophyta in the Mediterranean and Along the Israeli Shores"***

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Prof. Zvy Dubinsky in an emeritus Professor in Bar Ilan University, Ramat Gan, Israel. He obtained his Ph.D. from Bar Ilan University in 1978 and spent his postdoctoral fellowship at Queens College, NY, where he worked on the physiology and biochemistry of algal lipids and on their potential in biofuel production. His contributions in the field of photo-acclimation of phytoplankton and their interactions with the underwater light field have changed the field markedly. Subsequently, he added to his activities a strong interest in coral ecology and photobiology, formulating the first integrated views unifying energy and nutrient fluxes in zooxanthellate corals. These views were instrumental in understanding the detrimental effects of anthropogenic eutrophication on coral reefs. Additional projects were the ecophysiology of desert invertebrates and the physiology of mangroves. Dr. Dubinsky spent his Sabbaticals at Brookhaven National Laboratories, Rockefeller University, and three periods at the Research Center for Advanced Science and Technology, Tokyo University. He participated in several research expeditions to the coral reefs of Sinai, Antarctica, Eritrea, and the Seychelles, and worked on research projects at the Australian Institute of Marine Science, Hawaii Institute of Marine Biology, and the University of the Ryukyus, Okinawa. Professor Dubinsky has sponsored over 30 M.Sc. and 30 Ph.D. students and has published 200 scientific papers, edited the volume Coral Reefs (vol. 25, Ecosystems of the World, Elsevier 1990). He has organized, convened, and chaired several International symposia, workshops, and seminars, and is the recipient of several international and Israeli grants.

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INVASIVE AND ALIEN RHODOPHYTA IN THE MEDITERRANEAN AND ALONG THE ISRAELI SHORES

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1. Invasive and Alien Red Seaweeds in the Mediterranean

Hundreds of marine organisms including algae, phytoplankton, zooplankton, sponges, Cnidarians, mollusks, worms, arthropods, and fish species have already migrated via the Suez Canal in Egypt since the Grand Opening in November 1869. The Lessepsian migration takes place mostly from the Red Sea to the Mediterranean. However, few species migrated in the opposite direction (the Anti-Lessepsian migration) (Golani and Ben-Tuvia, 1995; Golani et al., 2002). The factors that facilitate the mainly northwardly transport to the Mediterranean were well described by Por (1971, 1978) who claims that high salinity and high temperature in the Levant Basin of the Mediterranean increase the chances of success of Red Sea invaders. The fact that the Red Sea contains far more species when compared with the temperate fauna in the Mediterranean also contributes to the predominance of Red Sea organisms among Lessepsian migrants. In addition, the high salinities of the two bitter lakes along the canal, reaching 48‰ (Morcos and Messieh, 1973), make the traverse harder for Mediterranean than for Red Sea species, since the latter is considerably more saline. The fact that Indo-Pacific organisms show more versatile adaptation to various ecological niches helps them to compete successfully with Mediterranean species and occupy vacant ecological niches as well (Por, 1971). Other important reasons to the northern transport are the winds and the northward water current leading from the southern end of the canal to its north. These streams support species migration as animal eggs, larvae, and juveniles, and probably algal spores as well (Madl, 1999). International maritime traffic also plays an important role (as a transportation vector) conveying marine species all around the world through the ships ballast water (Nehring, 2002, 2005; Streftaris et al., 2005) or attached to the ships' hulls (Verlaque et al., in press).

The significance of invasive species (also known as nonindigenous, exotic, introduced, alien, or nonnative species) in marine ecosystems worldwide has been highlighted in recent years. The scientific community discussed intensively the impact of invasive species from ecological and economical points of view

(Streftaris et al., 2005). Invasive species are generally recognized as a serious threat to environments and economies throughout the world (Wilcove et al., 1998; Dukes and Mooney, 1999; Pimental et al., 2000; Davis et al., 2001). These nonnative species can often rapidly adapt to the new environments (Huey et al., 2000; Mooney and Cleland, 2001), increase in abundance (Richardson et al., 2000; Kolar and Lodge, 2001; Colautti and MacIsaac, 2004), and play a conspicuous role in the recipient ecosystems (Boudouresque and Verlaque, 2002). These threaten native biological diversity (IUCN, 2002) replacing keystone species and/or being economically harmful (Boudouresque and Verlaque, 2002). Some of these species can sometimes even threat human health (EPA, 2001). However, invaders' success is far from being predictable (Boudouresque and Verlaque, 2002); only a small fraction of transported alien species become established, and of these generally only about 1% become pests (Williamson, 1996).

Since the beginning of the twentieth century, the number of alien marine macrophytes found in the Mediterranean has nearly doubled every 20 years (Boudouresque and Verlaque, 2002). Over 100 alien macrophytes were identified so far in the Mediterranean, of which 71 belong to the Rhodophyta, 22 Heterokontophyta (Phaeophyceae), 16 Chlorophyta, and 1 seagrass, belonging to the Magnoliophyta division (flowering plant) (Verlaque et al., in press). Nineteen of the invader species listed above caused obvious damage to biodiversity, fisheries, health, and/or infrastructure, and are defined as worst-invasive macroalgae (Streftaris and Zenetos, 2006).

Boudouresque's (2003) partial list of Mediterranean fauna and flora shows that the Mediterranean distribution data for the three main seaweed phyla are: Chlorophyta 16%, Rhodophyta 64%, and Heterokontophyta-Phaeophyceae 20% (Fig. 1a), which is very close to percentages established for the introduced seaweeds (Fig. 1b). Consequently, Rhodophyta do not appear to be much more efficient in introduction and invasive events than the two other groups. Thus, Rhodophyta contribute a fraction of the reported alien macrophytes entering the Mediterranean flora, very similar to that of their global distribution (Fig. 1). One wonders why their competitive edge in deep waters, discussed below, does not confer any advantage by providing invasion pathways unavailable to other taxa.

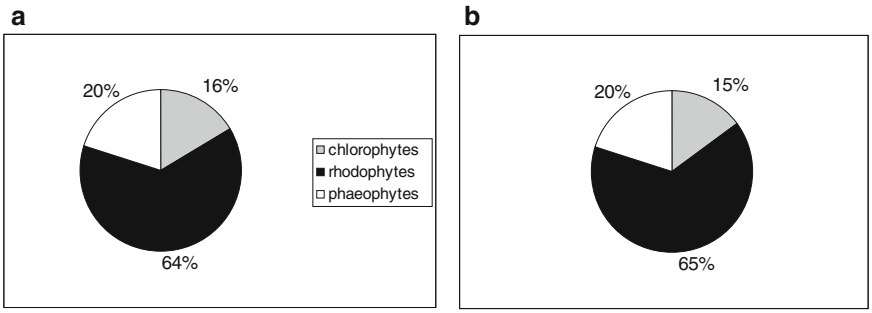

Figure 1. Distribution of seaweeds by phyla: **(a)** taxa in the Mediterranean **(b)** alien in the Mediterranean.

Rhodophyta, the red seaweeds, are unique macroalgae that share with the prokaryotic Cyanobacteria having phycobilins rather than chlorophylls as the main components of their photosynthetic antennae. In the Rhodophyta, R-phycoerythrin is the dominant light harvesting pigment and is responsible for the characteristic red-purplish hues of these algae. R-Phycoerythrin is a large 240 kDa red phycobiliprotein isolated from red algae. It has a strong absorption peak at 565 nm and a secondary absorption peak at 492 nm. These absorption peaks confer on these plants the capability of efficiently using green and blue light, which explains their dominance in the deeper parts of the photic zone. With depth, the red domain of the solar spectrum is attenuated, by both water- and chlorophyll-containing phytoplankton cells, resulting in a progressive increase in the blue and green components of sunlight (Graham and Wilcox, 2000).

Alien red seaweed species, which already invaded the Mediterranean (Table 1), mainly entered via the Suez Canal (Indo-Pacific origin) and sometimes through the Straits of Gibraltar (Atlantic origin) (Verlaque et al., in press;

Table 1. Alphabetic list of alien Rhodophyta taxa in the Mediterranean, with information about their origin (at for Atlantic species, IP for Indo-Pacific and Sh for Southern hemisphere) and status (A for alien species and E for established ones) (Verlaque et al., in press).

Taxon	Origin	Status
Acanthophora nayadiformis (Delile) (Papenfuss)	IP	E
Acrochaetium codicola (Børgesen)	IP	E
Acrochaetium robustum (Børgesen)	IP	A
Acrochaetium spathoglossi (Børgesen)	IP	A
Acrochaetium subseriatum (Børgesen)	IP	A
Acrothamnion preissii (Sonder) (E.M.Wollaston)	IP	E
Agardhiella subulata (C. Agardh) (Kraft and M.J. Wynne)	At/IP	E
Aglaothamnion feldmanniae (Halos)	At	E
Ahnfeltiopsis flabelliformis (Harvey) (Masuda)	IP	E
Anotrichium okamurae (Baldock)	IP	E
Antithamnion amphigeneum (A.J.K. Millar)	IP	E
Antithamnion nipponicum (Yamada and Inagak)	IP	E
Antithamnionella boergesenii (Cormaci and G. Furnari) (Athanasiadis)	IP	E
Antithamnionella elegans (Berthold) (J.H. Price and D.M. John)	IP	E
Antithamnionella spirographidis (Schiffner) (E.M. Wollaston)	IP	E
Antithamnionella sublittoralis (Setchell and Gardner) (Athanasiadis)	IP	E
Antithamnionella ternifolia (J.D. Hooker and Harvey) (Lyle)	IP	E
Apoglossum gregarium (E.Y. Dawson) (M.J. Wynne)	IP	E
Asparagopsis armata (Harvey)	IP	E
Asparagopsis taxiformis (Delile) (Trevisan de Saint-Léon)	IP	E
Bonnemaisonia hamifera (Hariot)	IP	E
Botryocladia madagascariensis (G. Feldmann)	IP	E
Ceramium bisporum (D.L. Ballantine)	At	A
Ceramium strobiliforme (G.W. Lawson and D.M. John)	At	A
Chondria coerulescens (J. Agardh) (Falkenberg)	At	E

(continued)

Table 1. (continued)

Taxon	Origin	Status
Chondria curvilineata (F.S. Collins and Hervey)	At	E
Chondria pygmaea (Garbary and Vandermeulen)	IP	E
Chondrus giganteus f. *flabellatus* (Mikami)	IP	E
Chrysymenia wrightii (Harvey) (Yamada)	IP	E
Dasya sessilis (Yamada)	IP	E
Dasysiphonia sp.	IP	E
Feldmannophycus okamurae (Yamada) (Mineur et al.)	IP	E
Galaxaura rugosa (J. Ellis and Solander) (J.V. Lamouroux)	IP	E
Ganonema farinosum (J.V. Lamouroux) (K.C. Fan and Y.C. Wang)	IP	E
Goniotrichiopsis sublittoralis (G.M. Smith)	IP	E
Gracilaria arcuata (Zanardini)	IP	A
Grateloupia asiatica (S. Kawaguchi and H.W. Wang)	IP	E
Grateloupia lanceolata (Okamura) (Kawaguchi)	IP	E
Grateloupia patens (Okamura) (S. Kawaguchi and H.W. Wang)	IP	A
Grateloupia subpectinata (Holmes)	IP	E
Grateloupia turuturu (Yamada)	IP	E
Griffithsia corallinoides (Linnaeus) (Trevisan)	At/IP	E
Herposiphonia parca (Setchell)	IP	E
Hypnea cornuta (Kützing) (J. Agardh)	IP	E
Hypnea flagelliformis (Greville ex J. Agardh)	IP	A
Hypnea spinella (C. Agardh) (Kützing)	At/IP	E
Hypnea valentiae (Turner) (Montagne)	IP	E
Laurencia caduciramulosa (Masuda and Kawaguchi)	IP	E
Laurencia okamurae (Yamada)	IP	E
Lithophyllum yessoense (Foslie)	IP	E
Lomentaria hakodatensis (Yendo)	IP	E
Lophocladia lallemandii (Montagne) (F. Schmitz)	IP	E
Nemalion vermiculare (Suringar)	IP	E
Neosiphonia harveyi (J. Bailey) (M.-S. Kim et al.)	IP	E
Nitophyllum stellato-corticatum (Okamura)	IP	E
Pleonosporium caribaeum (Børgesen) (R.E. Norris)	IP	E
Plocamium secundatum (Kützing) (Kützing)	Sh	E
Polysiphonia atlantica (Kapraun and J.N. Norris)	At	E
Polysiphonia fucoides (Hudson) Greville	At	E
Polysiphonia morrowii (Harvey)	IP	E
Polysiphonia paniculata (Montagne)	IP	A
Porphyra yezoensis (Ueda)	IP	E
Pterosiphonia tanakae (S. Uwai and M. Masuda)	IP	E
Rhodophysema georgei (Batters)	At/IP	A
Rhodymenia erythraea (Zanardini)	IP	A
Sarconema filiforme (Sonder) (Kylin)	IP	E
Sarconema scinaioides (Børgesen)	IP	A
Solieria dura (Zanardini) (F. Schmitz)	IP	A
Solieria filiformis (Kützing) (Gabrielson)	At	E
Symphyocladia marchantioides (Harvey) (Falkenberg)	IP	E
Womersleyella setacea (Hollenberg)	IP	E

Zenetos et al., 2005, 2008). However, seaweed invasion of the Mediterranean also occurred by accident such as in the famous case of the very aggressive and invasive green seaweed *Caulerpa taxifolia* (Chlorophyta), which was accidentally released to the Monaco shore, as a colony of 1 m^2, from the Oceanographic Museum of Monaco public aquaria in 1984 (Meinesz, 1999; Meinesz et al., 2001). This seaweed spread rapidly to France, Italy, Spain, Croatia, Tunisia (Streftaris and Zenetos, 2006), and probably to other Mediterranean shores. It is often called the "killer alga," since it caused very serious ecological problems by decreasing biodiversity dramatically in native marine ecosystems (Gravez et al., 2001).

Of the Rhodophyta species listed in Table 1, *Asparagopsis taxiformis*, *Asparagopsis armata*, *Acrothamnion preissii*, *Polysiphonia morrowii*, and *Womersleyella setacea* might become a serious problem in the Mediterranean and exhibit a strong invasive behavior; therefore, they are included in the list of the "Worst invasive alien species threatening biodiversity in Europe" (European Environmental Agency, 2007) and also in the list of "Alien marine species in the Mediterranean – the 100 'worst invasives' and their impact" (Streftaris and Zenetos, 2006).

2. Alien Red Seaweed Species in the Israeli Mediterranean

Published seaweed surveys along the Israeli shores are very rare. Therefore, there has been no proper monitoring and tracking of changes in red seaweed populations along the Israeli Mediterranean shores. Nevertheless, the few published surveys (Edelstein, 1960, 1962, 1964; Lipkin and Safriel, 1971; Lundberg, 1981, 1984, 1986, 1991, 1995, 1996; Hoffman, 2004) present a very interesting picture. Although the Israeli Mediterranean shore is near the northern end of Suez Canal in Egypt, which is the main source for Indo-Pacific alien species in the Mediterranean, only six alien red seaweed species (9.6% from all 62 alien Rhodophyta species reported from the Mediterranean) were reported from the region. Of these, *Acanthophora nayadiformis* (Fig. 2), *Hypnea cornuta*, and *Sarconema filiforme* (Fig. 3) became very common mainly in the intertidal zone and could be regarded as already established along the Israeli Mediterranean shores. However, *Asparagopsis taxiformis* and *Ganonema farinosum* (synonym of *Liagora farinosa*) were found as single specimens in the intertidal at Mikhmoret beach alone (Lipkin, 1962; Hoffman, 2004). This fact might indicate that their introduction is debatable.

3. *Galaxaura rugosa* in the Haifa Bay: Invasion?

Galaxaura rugosa (J. Ellis and Solander) J.V. Lamouroux 1816 (Fig. 4) is the sixth alien Rhodophyta of Indo-Pacific origin found in the Israeli Mediterranean. It was first described from the Israeli Mediterranean at the Haifa Bay during an

Figure 2. *Acanthophora nayadiformis* from the Israeli shores.

Figure 3. *Sarconema filiforme* from the Israeli shores; note the evolving oxygen bubbles.

Figure 4. *Galaxaura rugosa* from Haifa Bay.

autumn survey made in 2003. The specimen was found in the drift among other seaweeds washed ashore (Hoffman et al., 2008). The type was collected from Jamaica (probably as a gametophyte) (Papenfuss et al., 1982).

This red seaweed belongs to the family Galaxauraceae in the Nemaliales order (Wang et al., 2005) and typically consists of reddish hollow cylindrical and hairy branches, 1–4 mm in diameter, with yellowish or whitish pits at the ends. It forms a cushion structure, which is 8–15 cm long and 10–15 cm wide. However, according to Lipkin and Silva (2002), these algae might form clumps about 40 cm in diameter at the Dahlak Archipelago (Africa).

Galaxaura rugosa is very common on rocky foundations in the world oceans shores mainly around the equator. It is found in the Indian Ocean (Silva et al., 1996), the southern Pacific Ocean (Littler and Littler, 2003), and the Atlantic Ocean shores such as Morocco (Benhissoune et al., 2002), Cameroon (John et al., 2004), Mexico, and Brazil (Taylor, 1960). The southern distribution limit of this species appears to be in New Zealand (Adams, 1994) and Australia (Lewis, 1984; Millar and Kraft, 1993; Huisman, 2006) shores, while its northern distribution border is apparently limited to the Chinese shores (Tseng, 1984) and Japan islands (Yoshida, 1998). This seaweed was lately discovered in South Africa (De Clerck et al., 2002, 2005).

According to reports and herbarium catalogue indexes, *G. rugosa* individuals were collected in Israeli (Eilat), Jordanian, and Egyptian Red Sea shores during the twentieth century (Papenfuss, 1968).

This macroalga mainly grows on rocky substrates in the subtidal, lower intertidal (Huisman and Borowitzka, 1990; Huisman, 2006), midlittoral (Rodríguez-Prieto et al., 1999), and infralittoral (Lipkin and Silva, 2002; Mateo-Cid et al., 2006) zones. In Taiwan, it usually grows on rocky substrata at a depth between 0 and 10 m (Wang et al., 2005). When the foundation is a coral reef, this seaweed tends to grow on the corals (Littler and Littler, 2003).

Observing the seaweeds in the Haifa Bay drift mainly after winter storms, and exploring the intertidal and the infralittoral zones during the winters of 2005–2006, 2006–2007, and in the spring of 2007, we found that *G. rugosa* became one of the main components in the seaweed drift, mostly following wintertime storms when its quantities were estimated at 30% of the total (R. Hoffman, unpublished). These facts indicate that in the eastern Mediterranean, its vegetative reproduction (from fragments) occurs at the end of autumn and during wintertime. The main part of the thallus is released to the water by fragmentation process and only a small holdfast remains attached to the rocks. This small thallus will start to grow again during springtime. Fragmented individuals continue releasing spores (sexual reproduction) from sporangia during the winter. Thallus elongation and development from the alga base takes place during spring and summertime.

During our survey of *G. rugosa*'s habitats at the Haifa Bay during September 2006, we found that this macroalga appears sporadically and rarely on submerged rocks in the shallow waters (at about 50 cm depth below the datum line). However, it was more common on deeper rocks. Two meters below the datum line (and further below), it becomes one of the main components of the flora, along with *Sargassum acinarium* and *Codium* sp.

A study carried out in the Haifa Bay area 50 years ago documented the algal associations and their ecology and distribution on the sublittoral rocks (Edelstein, 1960, 1962, 1964).

Edelstein's survey took place in 13 deep sites (18–90 m deep). Algae samples were harvested and retrieved from the deep sea rocks to the Israeli ministry of agriculture's fishing boats by dredge and bottom grab. One hundred seaweed taxa were identified from the Bay, of which 56 belong to the Rhodophyta division. In that study, no evidence of *G. rugosa* in the Haifa Bay region was found. However, the congeneric *G. adriatica* Zanardini was documented as a common species, which was collected at a depth of 20 m. *G. adriatica* is currently regarded as synonym of *Tricleocarpa fragilis* (Linnaeus) Huisman and Townsend (1993) that is native in the Mediterranean Sea. Since the last published seaweed survey in the Haifa Bay took place over 50 years ago, we can only assume that *G. rugosa* settlement in the Haifa Bay occurred during the past 50 years.

Reports from Lebanese and Syrian shores (Bitar et al., 2000; Lakkis and Novel-Lakkis, 2000, 2001) claim that the first (published) observation of this seaweed in the Mediterranean Sea was in 1990 in Syria. Mayhoub (1990) identified *G. lapidescens* (J. Ellis and Solander) J. V. Lamouroux, which is currently regarded as synonym of *G. rugosa* in the Syrian coast. In 1999, Bitar et al. (2000) identified it in Ras Beirut region in Lebanon. According to the current survey

from the Haifa Bay and the surveys from Lebanon and Syria, *G. rugosa* tends to spread northward in the Mediterranean Sea. Since *G. rugosa* populations were found very close to marine ports, we hypothesize that its fragments and/or spores might have been transported from the Red Sea through the Suez Canal in ballast water and released accidentally near the Marine ports of Haifa (Israel), Beirut (Lebanon), Latkia, and Baniyas (Syria). However, owing to the political situation in the Middle East, there have been no marine trading relations between Israel and Lebanon or Syria for 60 years; hence, transportation vector has been minimal. Since the Levant basin is characterized by a northward direction of the current along its eastern shores, that flow is more likely to have acted as a transporting vehicle for *G. rugosa* propagules. Individuals or spores that settled on infralittoral rocks in the Eastern Mediterranean basin encountered similar abiotic conditions (such as high salinity and temperatures) as in the tropical region of the Red Sea, conditions that probably facilitated settlement.

As invasive species increase their abundance due to high proliferation (Colautti and MacIsaac, 2004) and play conspicuous role in recipient ecosystems (Boudouresque and Verlaque, 2002), we define *G. rugosa* as invasive species in the Haifa region. The present surveys show that *G. rugosa* became very common on the sublittoral rocks.

Its large abundance in the drift is conspicuous. Moreover, its large biomass and high proliferation on the upper surface of deep water rocks during the end of summer and autumn 2006 indicate that this seaweed already overtakes this habitat. As no former survey was published from the Haifa Bay since 50 years ago, we can only assume that this alga replaced the natural flora, which existed on the upper face of these rocks. Bitar et al. (2000) claimed that *G. rugosa* does not seem to act as an invasive species in Lebanon and Syria. However, surveys from Ibn Hani (Lattakia) in Syria indicated that *G. rugosa* covered most of the rocky substrates in the *Cymodocea nodosa* (seagrass) meadows at a depth of 12 m (Bitar et al., 2003). This indication implies that *G. rugosa* has become invasive and competitively superior on these rocks.

It is noteworthy to compare the cases of *Caulerpa racemosa* (Frosskål) J. Agardh (Chlorophyta) and *Stypopodium schimperi* (Buchinger ex Kützing) Verlaque & Boudouresque (Phaeophyceae) with that of *G. rugosa.* These are two other immigrant seaweeds species from the Red Sea, which were officially identified as invasive species in many locations in the Mediterranean (Streftaris and Zenetos, 2006). The former deeply changed phytobenthic community structure (Piazzi et al., 2001) and decreased seaweed diversity (Gravez et al., 2001) in many locations. Studies on the effects of *C. racemosa* on local flora showed that the total percentage cover and diversity were lower in invaded areas when compared with the control areas where *C. racemosa* was absent (Piazzi and Cinelli, 2003). *S. schimperi*, which is well known as invasive in the eastern Mediterranean basin, especially along the Levantine shores (Verlaque and Boudouresque, 1991) in Lebanon (Bitar et al., 2000, 2003) and Syria (Mayhoub and Billard, 1991), also spread to Libya, Egypt, Turkey (Bitar et al., 2000), and was even reported in the

Table 2. Invasion stages according to Colautti and MacIsaac (2004).

Stage	Specification
Stage 0	Propagules residing in a donor region
Stage I	Propagule introduction
Stage II	Propagule establishment
Stage III	Species localized and numerically rare
Stage IVa	Species widespread but rare
Stage IVb	Species localized but dominant
Stage V	Species widespread and dominant

Aegean Sea (Cocito et al., 2000). That species was lately washed ashore during spring in some locations along the Israeli beaches. In some cases, its fragments covered large areas on the beach and therefore it is suspected as becoming an invasive species. There is no doubt that the Lessepsian seaweed migrant *G. rugosa* has become an integral component among the indigenous local flora that covers the infralittoral rocks in the Haifa Bay. Moreover, its large quantities in the seaweeds drift during winter, and the fact that it covered many infralittoral rocks during summer suggests that it probably had displaced indigenous local flora. These two facts together show that *G. rugosa* became one of the dominant seaweeds in the survey region. Since becoming invasive is the climax of the process that begins with establishment and local spread, followed by increase in abundance (Richardson et al., 2000; Kolar and Lodge, 2001), we believe that *G. rugosa* can be indeed regarded as an invasive species.

The model of Colautti and MacIsaac (2004) describes the stages (Table 2) and filters by which alien species pass through from being as propagules residing in their donor region until they become widespread and dominant in the new acceptor settlement location, which they occupy. According to the results of the present survey (Hoffman et al., 2008) and other unpublished surveys done lately along the Israeli Mediterranean shore, we believe that *G. rugosa* has already reached stage IVb of that model, as it remains localized (found mostly in the Haifa region) but dominant (Colautti and MacIsaac, 2004).

We believe that in case *G. rugosa* does reach stage V, becoming widespread and dominant, it might cause long-term environmental changes. In this case, it might cause major changes in the community structure and function of the marine flora along Israel coast and therefore might cause damages to the local marine fauna.

4. Summary

Future seaweed studies should be focused on the effects of invasive nonindigenous seaweeds species such as *G. rugosa* and *S. schimperi* on the coastal ecosystems along the Israeli and Mediterranean coast, and their impact on fisheries and leisure sports. Monitoring the changes in these populations' distribution is important to estimate and analyze their effects on the local marine flora and fauna.

The spread of invasive species should be treated as one of the great historical convulsions in the world's fauna and flora (Elton, 1958), resulting from globalization and the associated dramatic increases in marine traffic. This problem is parallel to the spread of hitherto endemic diseases to faraway human communities due to the increase in human mobility.

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THE EXTREME ENVIRONMENTS OF *PORPHYRA*, A FAST GROWING AND EDIBLE RED MARINE MACROALGA

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1. Introduction

1.1. TAXONOMY

Porphyra (Rhodophyta, Bangiaceae) is a unique seaweed easily distinguished by its filamentous or leafy thalli (= also referred as blades or fronds) (Schneider and Wynne, 2007). *Porphyra* is represented by ca. 130 currently recognized species (Yoshida et al., 1997; Brodie and Zuccarello, 2007), which are particularly abundant in cold-temperate and boreal shores of the Northern and Southern Hemispheres. Few species can be found at the tropics or at the poles (Wiencke and Clayton, 1998). On the North European coasts and in the Mediterranean Sea, more than 20 species have been recorded. *Porphyra* has rather simple morphology, and discrimination between species by morphological characters is not easy to accomplish (Fig. 1). The characters currently employed include (1) number of cell layers and chloroplasts, (2) leaf margin, (3) shape of the blade, (4) structure of the basal part, (5) thickness of the blade, (6) color of the blade, and (7) sexuality (Kurogi, 1972; Yoshida, 1997). More recent molecular tools and approaches (i.e., plastid and nuclear molecular data and karyological observations) have helped clarifying species concepts and taxonomical relationships, all leading to a greater understanding of *Porphyra* geographical distribution worldwide (Nelson, et al., 2001; Brodie et al., 2007; Zuo et al., 2007).

1.2. REPRODUCTION AND LIFE CYCLE

Porphyra can reproduce both sexually and asexually, and presents a complex, exceptional life history. The so-called biphasic life cycle is composed of a diploid and haploid stage. The haploid phase, also termed gametophyte, is the foliose stage as seen growing in the intertidal zone (Fig. 1). Toward the end of the macrostage, generally as spring time arrives, the thallus goes through the sexual reproductive stage in which male and female gametes are produced by the haploid fronds to form the diploid phase, known as *conchocelis*-stage (sporophyte).

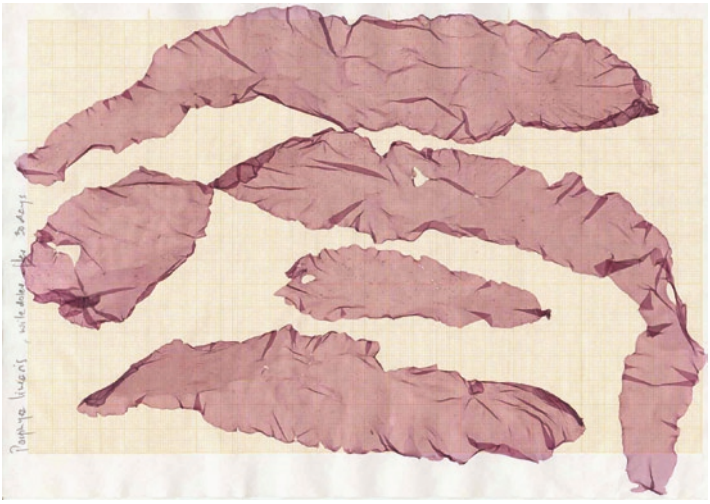

Figure 1. Dry-preserved, double-layered thalli (max length ca. 30 cm) of *Porphyra* collected in the eastern Mediterranean.

Factors triggering *conchocelis* formation are temperature and day-length. This form is microscopic and survives harsh conditions of temperature and irradiance within shells of bivalves or soft substrate within the intertidal. When conditions become appropriate on early winter, *conchocelis* will mature and release spores (i.e., conchospores) after meiotic division. In some species of *Porphyra* during the growing season of the foliose stage, asexual reproduction may take place by means of asexual spores (referred in the literature as monospores or archeospores, Nelson et al., 1999). The role of the asexual reproduction from blade to blade via asexual spores in nature is not clear but believed to enlarge the season of the gametophytes in the winter months. How *Porphyra* persisted through unfavorable seasons was not known for many years, even when *Porphyra* species were brought into cultivation. The complete life cycle was suggested by Drew (1949), and later confirmed by Japanese phycologists during the 1950s. An important factor contributing to the survival and spread of the genus *Porphyra* is the efficiency of its reproductive system, in particular the long fertile season and the ability of the conchocelis to survive and remain fertile for several seasons.

2. Physiological Features of *Porphyra* in Natural, Extreme Growth Environments

2.1. DESICCATION

Most *Porphyra* species are generally restricted to the high intertidal zone, in which the presence of other seaweed is rare. To survive in the upper intertidal belt, seaweeds must have high cellular ability to tolerate desiccation, and high capacity

Figure 2. *Porphyra* thriving in the upper intertidal during winter time in the eastern Mediterranean.

of photosynthetic recovery following air exposure (Lipkin et al., 1993). During desiccation, fronds may continue their photosynthesis at maximal rates until their relative water content (RWC) drops to only 60%. Moreover, net photosynthesis could still be positive even with only 10% RWC. Thus, desiccation tolerance is by far a key factor in the ability of *Porphyra* to occupy the high intertidal. Studies that have examined the photosynthetic response of *Porphyra* to desiccation have indicated a much greater capacity of recovery from a given degree of dehydration than other seaweeds located within the low intertidal (Abe et al., 2001). While typically winter species required low seawater temperatures and irradiances, during low tides the alga becomes overexposed to high light and temperature and severe desiccation can be frequent (Lipkin et al., 1993; Katz et al., 2000). Such adverse conditions can be overcome by algal self-shading and wave action more typical of winter days (Fig. 2). Since most *Porphyra* species thrive abundantly during winter time, a photoperiodic response of spore liberation from the *conchocelis*-stage to give fronds is apparent.

2.2. PHOTOACCLIMATION AND PHOTOINHIBITION

Porphyra is a seaweed constantly exposed to the effects of photoinhibition of photosynthesis, the process in which excessive visible light destroys key thylakoid membrane proteins that are not being replaced at the same pace as they are degraded (Herbert, 1990). Plants endemic to high irradiance environments are resistant to photoinhibition, and one remarkable example is *Porphyra*, which persists naturally under extreme photoinhibitory conditions in the high intertidal zone experiencing extreme desiccation under full sunlight on a daily basis during

exposure by low tides. *Porphyra* photoinhibition resistance is related to its ability to reduce the rate of photoinhibition both by means of biophysical processes (reducing heat effects on photosystem II) and increase stability of sensitive proteins within the photosystem II.

Typically, during acclimation to irradiance, seaweeds respond by changing the concentrations and ratios of their major photosynthetic pigments (Duarte and Ferreira, 1995; Talarico and Maranzana, 2000). In red seaweeds, photoacclimation to low irradiances results in increasing concentrations of chlorophyll *a* (chl *a*), phycoerythrin (PE), and phycocyanin (PC) within the light-harvesting complex. Photoacclimation in *Porphyra* is particularly rapid and may occur within hours when concentrations of PE, PC, and chl *a* increase significantly during morning hours and decrease toward the afternoon (López-Figueroa and Niell, 1990; Algarra et al., 1991; Rudiger and Figueroa, 1992; Figueroa et al., 1995). A longer-term adaptation to ambient light can be seen in the higher concentrations of PE from fronds of *Porphyra yezoensis* growing on the surface of algal mats when compared with levels of PE from algae growing underneath, in which irradiance levels are very low (Merrill et al., 1983). Generally, during acclimation to high irradiance PE levels in *Porphyra* species, decline to a much larger extent than those of chl *a*, and there is a sharp decrease in the density of phycobilisomes on the thylakoid. In addition, acclimation to low irradiance is concomitant to decreased cell size to favor light capture, since for cells having similar quantities of photosynthetic pigments the smaller ones absorb more light than the bigger ones on a surface basis (Dubinsky, 1992; Schanz et al., 1997). Thus, a photoprotective role of phycobilins against excessive irradiance, as seen by high PE/chl a ratios, has therefore been indicated by Talarico and Maranzana (2000) in red macroalgae and by Figueroa et al. (1997) in cryptomonas, probably through PE aggregates not attached to the phycobilisomes. Photosynthetically functional PE attached to the phycobilisomes at high irradiance (including high UV radiation) can lead to increased oxidative damage; however, unattached PE can act as UV screen substances and reduce chlorophyll damage. Decreased respiration rates, maintenance of relatively high photosynthetic rates, and low light compensation points are common responses of *Porphyra* species to low irradiance (Levitt and Bolton, 1991; Markager, 1993). These attributes together with the typically low epiphytic activity on *Porphyra* species create favorable conditions for cultivation in the laboratory (Katz et al., 2000) or in tanks (Israel et al., 2006) and can have ecological advantage in natural habitats. For example, *P. leucosticta* thrives in the Eastern Mediterranean and shores of Israel only in the winter. It occupies the uppermost belt of the intertidal zone without apparent competitors and shows high adaptability to varying irradiances (Katz et al., 2000). In spite of the characteristically low seawater temperatures and irradiance of winter time, *P. leucosticta* is commonly exposed at low tide to irradiances above its optimal range (about 70 μmol m^{-2} s^{-1}; Katz et al., 2000). Summer temperatures and irradiance levels are far too high for growth of this alga when it survives as the cryptic conchocelis phase in sheltered microniches. Therefore, survival strategies may

involve a combination of "resorting" of the thalli through wave action and tide change, and/or shielding of the main bulk of the plant by the upper blades. In this way, the thalli underneath are protected from desiccation and photodynamic damage.

2.3. NUTRIENT UPTAKE

Porphyra species are highly productive due to their thin-bladed thalli (high surface area to volume ratios), which favors rapid absorption of nutrients (Zavodnik, 1987; Levitt and Bolton, 1991; Stirk et al., 1995; Pedersen et al., 2004; Pereira et al., 2008) and maximizes light utilization by photosynthetic pigments (Lipkin et al., 1993; Israel et al., 1999; Katz et al., 2000). Specifically, the notable high capacity for nutrient uptake, particularly ammonium (NH4+) and nitrate (NO3–) of *Porphyra* offers a unique opportunity for its use in bioremediation and Integrated Multi-Trophic Aquaculture applications (Chopin et al., 2001; Chopin, 2006). The efficacy among *Porphyra* species as nutrient scrubbers should de compared to choose the right strains for bioremediation. Encouraging preliminary studies have demonstrated that some *Porphyra* species respond to high nutrient loads in areas of salmon and bivalves aquaculture by incorporating large concentration of nutrients into their tissues (Chopin et al., 2001).

2.4. TEMPERATURE

Temperature effects on growth capacity can be very strict, with optimal seawater temperatures between 10°C and 17°C to attain maximal yields in most species. Biomass yields of cultured *Porphyra* decreased substantially, five- to tenfold, when temperatures reach 18°C, with almost no growth observed at above 20°C (Katz et al., 2000, Yamamoto et al., 1991). Thus, as expected from the characteristic seasonal dynamics of *Porphyra* in nature, namely, active growth during cold months, temperature is a crucial limiting factor for cultivation, both in the sea or inland using in tanks and ponds. Few species, however, do thrive in more extreme temperatures. For example, *Porphyra vietnamensis* blades grow at 32°C under lab conditions (although with abnormal thallus morphology), and archeospores and conchospores developed satisfactorily at 30°C. Polar species develop normally at harsh temperatures of 0–2°C such as for *Porphyra endiviifolium* in the Antarctica during summer months with photoperiods of 20 h. Temperature is a key factor in *Porphyra* cultivation. *Porphyra* cultivation on nets in Asian latitudes begins when seawater temperature falls to 23–22°C in the autumn. Seawater temperature further falls and ranges from approximately 15 to 20°C at the end of the period of making nursery nets, when some of the nets are stored in refrigerators as frozen seed nets, and others are cultivated in the sea for the first harvest (Yamamoto et al., 1991). While *in vitro* optimal temperatures are around 15°C, nori cultivation farms in the sea grows optimally in the 8–10°C range (Yamamoto et al., 1991).

3. Cellular and Genetic Aspects of *Porphyra*

Genetic and molecular research carried out during the last decade on marine macroalgae has been clearly focused toward two major objectives. One is the taxonomical challenge of seaweeds in general, including *Porphyra*. Molecular techniques are common for the identification of economically important cultivars (Niwa and Aruga, 2006), and describe their phylogenetic relationship between Rhodophytes and other eukaryotic phyla. The second main objective of molecular and genetic research is to develop tools to improve the culture of economically important *Porphyra* strains. The cultivation technology of *Porphyra* is focused nowadays on improving the plant material on strain selection. Strains with late maturation and archeospore production are desirable, since the foliose economical important phase can be maintained over time. Several options are available for seed or seedling production intended for cultivation (both in the sea- or land-based) purposes (Dai et al., 2004). Low-costs production of protoplasts and electrofusion of protoplasts to improve quality of cultivars of *Porphyra* has been explored as a possible alternative that could potentially benefit the aquaculture industry (Dipakkore et al., 2005; Mizukami et al., 1995). For example, protoplasts of *P. linearis* (a species characterized by having large frond size) were combined with those of *P. miniata* (with superior taste and texture characteristics) and developed into plantlets of a few cells (Chen et al., 1995). Further, fusion of protoplasts between distant species (e.g., protoplasts of the green alga *Monostroma* and those of *Porphyra yezoensis*) has been investigated for breed selection (Kito et al., 1998), and tissue culture and 1–2 celled excised tissue of several *Porphyra* species can develop directly into blades (Notoya, 1999).

4. Economic Aspects of *Porphyra*

Among seaweeds, *Porphyra* species account for one of the largest source of food in the world market. *Porphyra*, known by the more common names of nori and laver, is dried and processed into thin purplish-black sheets. One of its common uses is in Japanese sushi, where it is wrapped on the outside of a small handful of soured, boiled rice topped with a piece of raw fish. *Porphyra* is also marketed in more simple forms such as dried, compacted packages in local markets in the Philippines, China, and Chile. *Porphyra* has been cultivated in Japan and the Republic of Korea since the seventeenth century, but even at that time production was insufficient to meet demands (Mumford and Miura, 1998). Cultivation was developed intuitively, by observing the seasonal appearance of spores, but *Porphyra* has a complex life cycle that was not understood until the 1950s (Drew, 1949). Since then, cultivation has flourished, and today the supply is virtually all from cultivation, which is conducted on a large scale in Japan, China, and the Republic of Korea. In 1999, the combined production from these three countries was just over 1.4 million metric tons produced in 2004 (FAO, 2006). It has the highest value of any cultivated

seaweed, about US$ 1,200 per wet ton. For comparison, the brown seaweeds used as food are valued at US$ 610/wet tons for *Laminaria* and US$ 530/wet tons for *Undaria* (McHugh, 2003). Beyond seaweed culinary trends developing during the last decades, *Porphyra* is a precious seaweed owing to its nutritional values and health merits. For example, 100 g dry weight *Porphyra* may contain protein levels as high as 40% (Marsham et al., 2007) while vitamin contents such as A, B12, B3, and C are all well above those defined as daily recommended dosage for humans (Yamada et al., 1999). Minerals such as Ca, Zn, and Fe are higher than traditional mineral-rich food like almonds, soybeans, and liver (Subba Rao et al., 2007).

4.1. CULTIVATION

Out of the approximately 130 identified species of *Porphyra*, only a few have served as commercial, sea-vegetable foods. Several variants of these naturally occurring species have been produced to enhance yields and culinary parameters of nori production in Japan, Korea, and China. Until the late 1980s, production of nori was almost equally balanced with consumption (Miura and Aruga, 1987). Rapidly expanding seaweed markets and degradation of marine environments have both led to steadily increasing demands for nori worldwide (McHugh, 2003; Merrill, 1993). Traditionally, *Porphyra* is cultivated directly in the sea by means of seeded nets, which are conveniently attached slightly below seawater levels, as shown in Fig. 3. The most frequently used species are *P. yezoensis* and *P. tenera*, yet several locally derived variants with fast growth rates and appropriate texture and color have been selected with time.

Figure 3. Traditional cultivation of *Porphyra* using seeded nets deployed just below seawater level.

Recently, land-based (tank and pond) seaweed cultivation has become popular and suggested to have several advantages over traditional, open-sea aquaculture such as that of *Porphyra*. In tanks, algal growth can to some extent be manipulated and seaweeds can be enriched with desired biochemicals. Abrupt pollution events may become detrimental for cultivation in the open sea. This and other environmental factors during the growth season make biomass yields unpredictable. Nevertheless, few studies have reported successful, sustainable cultivation of seaweeds in land-based tanks or ponds, and even fewer have described tank cultivation for *Porphyra* (Israel et al., 2006). In fact, it is very likely that tank cultivation is more common than reported in the scientific literature, as seaweeds have gained crucial roles in, for example, developing integrated aquaculture (Chopin et al., 2001; Fei, 2004). Mencher et al. (1983) described the use of ocean thermal energy conversion effluents to cultivate *Porphyra* in 1 m^3 tank compartments. Yamamoto et al. (1991) tested outdoor raceways as an alternative cultivation approach to grow *P. yezoensis* in Japan. Hafting (1999a, b) demonstrated monospore production, after cutting and maceration of foliose thalli as seeds, to establish a tank cultivation technology for *Porphyra*, while Notoya (1999) also proposed seed production from tissue culture of both monoecious and dioecious species.

Porphyra yezoensis, *P. tenera*, and *Porphyra* sp. have fast growth in the new habitat created by tanks and ponds (Friedlander and Levy, 1995; Israel et al., 1999). Intrinsic resistance to epiphytes and/or bacterial infections adds to successful tank cultivation. High productivities can be explained by a number of factors. First, cultures of free-floating blades are kept in constant motion by agitation (aeration) of the medium, resulting in more efficient use of nutrients, since agitation reduces boundary layers and prevents diffusion rates from limiting growth (Hafting, 1999a, b). Second, blades receive equivalent illumination with potentially higher production than in ocean-based net cultivation (Hafting, 1999a). Third, by regulating seawater exchange rates and seaweed density, tank cultivation may prevent the negative effects of epiphytes. Fourth, pond culture can be used for six to eight cycles of 2–3 weeks each, during the growth season. Another significant advantage of pond cultivation is the ability to control mineral nutrition resulting in the manipulation of organic and inorganic components of *Porphyra*. Productivities of *Porphyra* species in tanks are 50–300% higher than yields indicated for crops grown on seeded nets (Tanaka et al., 1997). All technologies for *Porphyra* cultivation would need to produce large amount of spores, which require storage. Liquid N_2 is the optimal cryopreservation agent since it controls physiological and genetic modifications to a maximum. Cryopreservation of cells and meristems of higher plants in liquid N_2 is an important tool for preservation of germplasm without genetic alteration. Cryopreservation of seaweeds in liquid N_2 has began in recent years and several examples on *Porphyra* preservation can be found in the literature (Fujiyoshi, 1997; Kuwano et al., 1996). There might be at least about 50 strains of *Porphyra* cryopreserved by the Japanese government as part of a nationwide genetic resource preservation project.

As opposed to plants, *Porphyra* has no suitable stage for preservation as for higher plants and can be preserved in both its foliose and filamentous form. Long-term preservation of up to about 1 year is possible taking into consideration some precautions: partial dehydration of the tissue is critical as it avoids intracellular freezing, which leads to vitrification and cell death, thus resulting in viable cytoplasm after thawing the thalli.

5. *Porphyra* in Globally Changing Environments

Global changes predicted for the marine environment include higher total Ci concentrations in seawater due to a continuous rise in atmospheric CO2 (predicted to double within the next century; Conway and Tans, 1996), a "greenhouse effect" created by specific gases, particularly CO2, trapping heat, which might increase the seawater temperature by an average of 3°C, and an increase in solar flux of UV radiation in its two damaging forms, UVA (320–400 nm) and UVB (290–320 nm) (Crutzen, 1992). While UV flux on the earth's surface has undergone significant reduction in the last decade, it is expected that in approximately 50–100 years time, the oceans and seas of the world could likely become more acidic, richer in inorganic carbon, and slightly warmer than they are today. These global changes will certainly place further environmental constrains on seaweed communities also, and will be of particular stress to seaweeds in the high intertidal zones.

Although restricted to coastal areas, benthic macroalgae play an important role in marine primary production. Also, they serve as food source for herbivores and detrivores, nursery areas for juvenile fish and crustaceans, and food stuff and production of natural products for humans. In addition, marine vegetation interacts with its environment and other marine organisms by buffering against large changes in nutrient concentrations. Despite the clear importance of marine macroalgae in coastal systems effects of rising Ci in seawater and UV radiation in nutrient-rich areas are fundamentally missing, particularly for seaweeds (Franklin and Forster, 1997; Israel and Hophy, 2002). Both UVA and UVB wavelengths can penetrate to ecologically significant depths in seawater with the absorption of UV radiation largely dependent on the concentration of chlorophyll and dissolved organic matter (Jerlov, 1950; Smith and Baker, 1979, 1989; Wood, 1987). It has been conventionally assumed that UVB radiation produces the greatest damage to living organisms (although one "positive" effect known so far is the increased availability of Fe in marine plants; Palenik et al., 1991); however, there is increasing, yet inconsistent, evidence that UVA by itself or combined with UVB may be less harmful than expected.

An increasing number of studies have shown that macroalgae exposed to deleterious UV radiation may suffer damage of the photosynthetic apparatus and, under severe UV stress, DNA damage (Franklin and Forster, 1997). Even under "normal" conditions, solar UVB is known to have inhibitory effects on photosynthetic performance and nutrient uptake (Larkum and Wood, 1993).

It further seems that the response to UVB is species-specific and that many organisms have evolved strategies such as photorepair or the presence of screening compounds for coping with deleterious effects of UVB radiation. Many intertidal macroalgae show less inhibition of photosynthesis by UVB than their subtidal counterparts (Wood, 1987; Gomez and Figueroa, 1998), clearly because differences exist between macroalgae living deep in shaded environments and those regularly exposed to surface or near-surface levels of radiation. Thus, production of UV-blocking agents (such as mycosporine-like amino acids-MAAs) in algae exposed to excessive UV levels are more commonly found for intertidal species (Gröniger et al., 1999; Sinha et al., 2000) and confer an important strategy to lessen UV photodamage. Thallus morphology can also influence the susceptibility of marine macroalgae to UV radiation, with thicker thalli being less sensitive than thinner thalli, although exceptions such as the leaf-like red alga *Porphyra* do exist (Gröniger et al., 1999).

Global warming forecasts rising sea levels as well. Thus, seawater temperature levels and sea-level trends seem to be correlated (Cabanes et al., 2001). Sea level has risen more than 120 m since the peak of the last ice age, about 18,000 years ago. The bulk of that rise occurred about 6,000 years ago. From 3,000 years ago to the start of the nineteenth century, sea levels have risen at a constant rate of 0.1–0.2 mm year^{-1}. Since 1900, the level has risen at 1–3 mm year^{-1} and rates are increasing by about 3 mm year^{-1} (Cabanes et al., 2001). This rapid rising should be regarded as a warning sign of the effect of global warming on sea levels. The impact of rising sea level is important because the most likely scenario involves the intertidal zone, the habitat occupied by most *Porphyra* species becoming permanently submerged. It has been indicated that air exposure in *Porphyra* is important physiologically to maintain high productivities. Controlled dehydration in *Porphyra* farming (even on a daily basis) is a common practice to accelerate growth and avoid epiphytes. Consequently, the dynamics and composition of algal communities on today's intertidal zone will be different when shifting temperatures, light intensities, and Ci create a different competitive environment for both intertidal and subtidal seaweeds. The impact of such predicted changes on *Porphyra* is yet to be determined.

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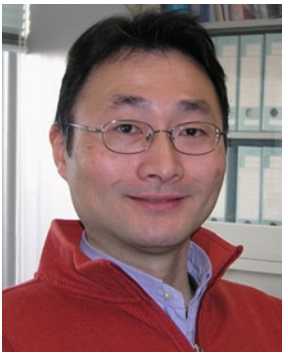

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INVESTIGATIONS ON REPRODUCTIVE AFFINITIES IN RED ALGAE

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1. Introduction

Allopatric speciation is the most widely accepted model proposed for speciation: once a population is divided by extrinsic barriers, genetic flow is interrupted between these disjunct subpopulations, and reproductive isolation is established as a by-product of the accumulation of genetic changes in these isolated populations. According to the biological species concept (Mayr, 1942), the evolution of reproductive isolation is a defining characteristic of speciation, and reproductive isolation contributes to the diversification of species by creating genetically independent lineages. It had been generally thought that isolation is more difficult in marine populations, with frequent gene flow over large distances (Hoffmann, 1987; van den Hoek, 1987; Norton, 1992; Shanks et al., 2003), as there seem to be far fewer extrinsic barriers in marine environments than in terrestrial ones (Palumbi, 1994). Such attributes are considered to limit the isolation of a species into allopatric populations less frequently, making allopatric speciation rarer (Mayr, 1954). However, recent molecular analyses have revealed great genetic divergences among/within populations in various marine organisms, including macroalgae, and thus, the generalization that speciation must be rare in marine habitats appears to be incorrect.

Since the mid-twentieth century, crossing studies have been carried out in various macroalgal groups to supplement morphological and cytological data, and these results have contributed to delineation of species boundaries, inference of evolutionary relationships, and disclosure of intraspecific diversities (cf. Mathieson et al., 1981; Guiry, 1992; Brodie and Zuccarello, 2006). However, in recent years, these processes are more effectively performed using molecular phylogenetic analyses that were developed in the last 20 years, and, in contrast, crossing studies are performed on only limited taxa. Some biologists consider that such crossing investigations provide more restricted information for taxonomic and phylogenetic studies, and because of the time-consuming efforts required, they are forced to forsake these "less fruitful" endeavors to concentrate on the molecular data in studying macroalgal speciation. However, it is highly debatable that information about reproductive affinities has little use and does not contribute to our understanding of algal evolution anymore. Can we fully explain the patterns and processes

of macroalgal speciation with only molecular evidence? Are not the reproductive affinities helpful except for macroalgal taxonomy? To answer these questions, it is necessary to demonstrate how the crossability information helps our understanding of macroalgal speciation and will contribute to further investigations on the genetic mechanisms of algal evolution.

The number of known red algal species (ca. 6,000 species) is much greater than green (ca. 1,100 species) and brown algae (ca. 1,500 species) (Graham and Wilcox, 2000). It is worthwhile to clarify how red algae diversify in their habitats and how they adapt to the circumstances under natural selection. Red algae, especially florideophycean algae, show a life history rather different from other macroalgae. Nonflagellate spermatia from male gametophytes attach to receptive trichogynes, hair-like cytoplasmic extensions of the female egg (carpogonium) (Fig. 1), and the zygote grows into a microscopic diploid structure on the female thallus. This diploid tissue, carposporophyte, is embedded in the parental female thallus, and the combination of the carposporophyte tissue and the surrounding female gametophytic tissue (pericarp) is called cystocarp (Fig. 2). Carpospores released from the carposporophyte grow into diploid sporophytes (tetrasporophytes), and their meiospores (tetraspores) develop into haploid gametophytes. This unique triphasic life history is thought to enhance reproductive fecundity and serve as an evolutionary compensation for loss of flagella (Searles, 1980; Brawley and Johnson, 1992).

Reproductive affinities have been investigated in the widespread taxa of red algae, because of the following advantages for crossing experiments. First, many

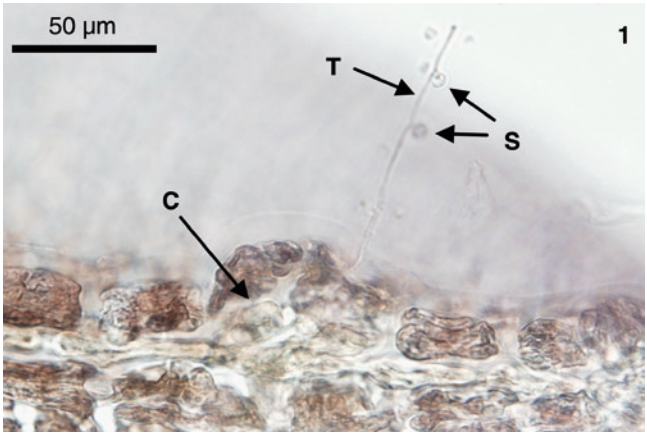

Figure 1. Female reproductive structure of *Caloglossa leprieurii*. Nonflagellate spermatia (S) from a male gametophyte attached to a trichogyne (T), hair-like cytoplasmic extension of the female egg, carpogonium (C).

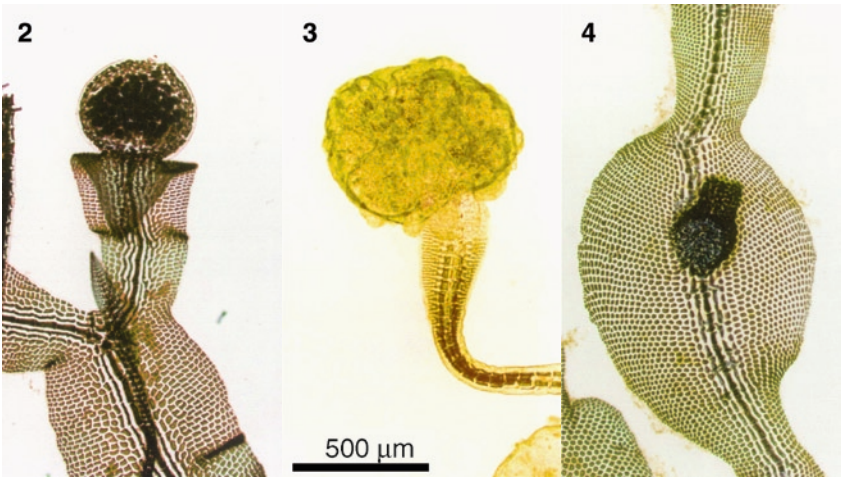

Figure 2-4. Various cystocarps produced in the artificial crosses of *Caloglossa leprieurii*. **2.** Normal cystocarp containing many carpsporangia inside. **3.** Abortive cystocarp that failed to discharge corpospores. **4.** Pseudocystocarp with few carposporophyte tissues inside.

algae require certain biotic and/or abiotic factors for gametogenesis and mating. For example, *Closterium* and *Volvox* need the presence of the opposite mating type to induce gametogenesis (Starr and Jaenicke, 1974; Tsuchikane et al., 2003). Gamete interaction requires a certain minimal gamete concentration (Nečas, 1981; Togashi and Cox, 2004), and a certain similar proportion of the two gamete types may be necessary for successful zygote formation (Richards and Sommerfeld, 1974). Synchronous gamete release is also crucial for successful fertilization of flagellated male and female gametes (see Santelices, 2002). In contrast, red algal gametogenesis is relatively frequent and successive at least in laboratory cultures, and hence, artificial hybridization can be carried out by putting fertile male and female thalli together, without any induction for gametogenesis and mating (e.g., Kamiya et al., 1997). Second, parthenogenetic development of gametes, which is common in green and brown algae (De Wreede and Klinger, 1988), is rare in red algae, except for apomictic entities of *Ahnfeltiopsis* and *Mastocarpus* (Polanshek and West, 1977; West et al., 1978; Guiry and West, 1983; Masuda et al., 1984, 1987); hence, it is easy to infer fertilization through the production of carposporophytes.

As the taxonomic implications of reproductive compatibility were well documented by Mathieson et al. (1981) and Guiry (1992), in this chapter, we will focus on the reproductive affinities, to provide useful information about the pattern and process of red algal speciation. We will first document the variations

of reproductive fertility in intra- or interspecific crosses, and then elucidate how the degree reproductive affinity is coupled with geographic distances, physiological differences, and/or genetic variations. Lastly, we will discuss the perspective and importance in assessing reproductive affinity for future research into algal speciation.

2. Prezygotic Isolating Mechanism

Reproductive isolation is achieved in a variety of ways and falls into two broad categories: prezygotic isolating mechanisms that act to prevent the formation of hybrid zygotes, and postzygotic isolating mechanisms that bring about reproductive failure at various stages after fertilization. In marine organisms, the simplest and probably the most common prezygotic mechanism is spatial separation that prevents genetic exchange between potentially interbreeding groups (see below for detail). Even if potentially interbreeding groups are not geographically separated, reproductive isolation can be maintained by ecological, temporal, or biochemical differentiation between them. Some of these prezygotic isolating mechanisms may operate simultaneously, and can make reproductive isolation more effective. As red algae are the only group of macroalgae completely lacking the flagellated stages, their fertilization is a random collision of two gametes, and as a result, some spermatia may end up on the trichogynes of incompatible females. Therefore, prezygotic isolating mechanisms are important to avoid a potential loss of fitness producing abortive hybrid progenies.

2.1. ECOLOGICAL ISOLATION

In ecological isolation, even if potentially interbreeding groups live in the same geographical area, they occupy different habitats and thus do not come into contact with each other. However, the effect of this isolation mechanism is considered to be limited in macroalgae, because free-living gametes are possibly exchanged between the habitats. Richerd et al. (1993) performed crosses between *Gracilaria verrucosa* (currently *G. gracilis*) collected from high and low tidal level and demonstrated that the fertility between the different tidal levels was not significantly different from that within the same tidal level. Recently, Engel et al. (2004) compared the population structure of *G. gracilis* in light of the spatial positions of populations with respect to height on the shore by analyzing seven microsatellite loci. They indicated that high-shore individuals were more frequently restricted to their original intertidal pools than low-shore individuals, and that height on the shore significantly influenced migration among pools. High-shore populations were found to experience longer periods of isolation from other intertidal pools at the same shore, and as a result, low-shore populations were found to be more open

to migrants (Engel and Destombe, 2002; Engel et al., 2004). Such asymmetrical gene flow between high- and low-shore populations can advance adaptation to local ecological conditions. As the environmental conditions, such as light quality and quantity, temperature, exposure time to air, and/or degree of wave exposure, differ with coastal depth, the difference in growth depth may possibly develop into ecological isolation. The Baltic Sea and adjacent regions provide a good example of how salinity gradient is effective in interrupting gene flow. The salinity gradient is created by a large inflow of freshwater into the Baltic Sea from over 200 rivers, in combination with the semi-enclosed geographical position of the Baltic basin (Bergström and Carlsson, 1994). In addition, this salinity gradient is stable year-round owing to the long water turnover times. Despite the Baltic Sea flora having developed from postglacial immigrants (8,000–4,000 BP), recent researches indicate great reproductive and genetic diversities of several macroalgae within this region (see Johannesson and André, 2006).

For example, *Ceramium tenuicorne* in the Baltic Sea and adjacent regions has been well investigated based on reproductive crossability, salinity tolerance, and genetic diversity. Many scientific names had been assigned to this alga owing to its morphological variability, and Gabrielsen et al. (2003) suggested that the specimens from the Skagerrak–Baltic region belong to the same species, owing to their interfertility and almost identical sequences of the nuclear ribosomal internal transcribed spacer 2 (ITS2) and the plastid Rubisco spacer region. However, the mitochondrial cox2-3 spacer analyses revealed five haplotypes in *C. tenuicorne* from the Skagerrak–Baltic region; one of them was restricted in the highest salinity site (Oslofjorden) where no other haplotype was detected, and another occupied the low (Baltic) and medium (Kattegat) salinity sites (Gabrielsen et al., 2002). In contrast to the cox2-3 spacer results, the genetic analyses of random amplified polymorphic DNAs (RAPDs) suggested a continuous cline corresponding to the salinity gradient (Gabrielsen et al., 2002). The authors imply that this incongruence of the two molecular data sets is possibly associated with the difference in the nuclear and mitochondrial inheritance, and also with the vicariant event during the glacial period. High genetic diversity has also been reported in other macroalgae (van Oppen et al., 1995b; Valatka et al., 2000; Coyer et al., 2003; Johansson et al., 2003) as well as seagrass (Reusch et al., 2000; Reusch, 2002; Olsen et al., 2004) distributed in the Baltic region. Although many Baltic macroalgae are considered to have been introduced from the North Atlantic after the last glacial maximum, populations inhabiting the Baltic have been influenced by different evolutionary forces including severe bottlenecks, genetic isolation, and strong selection in this novel habitat (Johannesson and André, 2006).

Physiological differentiation is observed in *Ceramium tenuicorne* from the Skagerrak–Baltic region: the isolates from low and high salinity sites showed different growth responses in different salinities, which corresponded to the salinity regimes of their original habitats, and their hybrid indicated an intermediate

pattern relative to that of the parents (Rueness, 1978; Rueness and Kornfeldt, 1992; Bergström and Kautsky, 2006). Bergström et al. (2003) observed a strong reduction in sexual reproduction and an increased tetrasporophyte dominance along the salinity gradient in *C. tenuicorne* from its inner distribution limit in the Baltic Sea. Although it is unknown whether this reproductive strategy is genetically fixed in these entities, it is presumed to affect the genetic exchange along the Baltic coasts.

A similar genetic disjunction between the eastern North Atlantic and Baltic Sea coasts was observed in *Phycodrys rubens*. Two genetic groups were recognized around these regions based on allozymes and RAPDs data: each genetic group occupied the European outer coasts and the North Sea/Baltic coasts, respectively, and these two genetic groups co-occurred in the Skagerrak and Kattegat regions (van Oppen et al., 1995a, b). As salinities decreased below 20 psu, the relative growth rate of marine strains of *P. rubens* from the North Sea was severely reduced when compared with the brackish strains from the Baltic Sea (Rietema, 1991). Ecotypic differentiations in salinity response have been observed in many other macroalgae distributed around this region (Russell, 1985, 1994; Thomas et al., 1990; Bäck et al., 1992; Rietema, 1993, 1995; Kristiansen et al., 1994; Serrão et al., 1996), and it is highly possible that this salinity gradient could be responsible for generating adaptive differentiation as well as may act as a barrier for gene flow among these ecotypes (Middelboe et al., 1997).

Difference in upper or lower temperature tolerance is also an important factor for algal ecological isolation. In the case of *Chondrus crispus* from two sites of Atlantic Canada, the strain from Bay of Fundy indicated a faster growth rate at 10–20°C than the strain from Gulf of St. Lawrence (Chen and Taylor, 1980). Cystocarps were produced on the female from the Gulf of St. Lawrence in the presence of the male from the Bay of Fundy, whereas no reaction was seen in the reciprocal cross. Their different temperature response may maintain "habitat segregation," and as a result, may restrict the genetic exchange between these localities, though Chopin et al. (1996) suggested low genetic diversities of *Chondrus crispus* around the Atlantic Canada. The difference in the upper or lower temperature for maturation within the same species, which have been reported in many kinds of red algae (e.g., Molenaar and Breeman, 1994; Molenaar et al., 1996; West et al., 1996), may also be an important factor for ecological isolation.

2.2. TEMPORAL ISOLATION

Temporal isolation is similar to ecological isolation, but with the separation occurring in time rather than space; the entities reproduce in different seasons or even different times of the day. Currently, there is one good instance for temporal

isolation of red algae. The western Pacific strains of *Chondrus ocellatus* show different photoperiodic response: the strains from Korea, China, and some Japanese sites form gametangia under long-day conditions, whereas those from two other Japanese sites (Enoshima and Sumoto) require short-day conditions for gametogenesis (Brodie et al., 1993). The photoperiodically different strains are interfertile once they produce gametes under their appropriate condition, and the responses to daylength in their F_1 gametophytes suggest Mendelian inheritance of this photoperiodic property.

2.3. BIOCHEMICAL ISOLATION

On biochemical isolation, it was found that the entities failed to fertilize successfully because of phenotypic incompatibilities. In the case of red algae, sexual reproduction is brought about by the attachment of spermatia to trichogynes. After the localized cell-wall breakdown of the trichogyne, the spermatial nuclei are transported along the trichogyne into the carpogonium, where karyogamy takes place (Mine and Tatewaki, 1994; Pickett-Heaps and West, 1998). Magruder (1984) demonstrated that spermatia from *Aglaothamnion cordatum* (as *A. neglectum*) specifically bind with trichogynes and hairs of female thalli, and that fimbriate cone-shaped appendages projecting from each end of the spermatium are responsible for the initial binding with trichogynes. The lectin-/carbohydrates-binding experiments of Ceramiacean species suggested that the attachment of spermatia to trichogynes is mediated by particular carbohydrates on spermatial surface and complementary carbohydrates-binding receptors on trichogynes (Kim and Fritz, 1993; Kim et al., 1996; Kim and Kim, 1999). In *Antithamnion sparsum,* d-mannose and l-fucose were involved in gamete recognition, whereas the latter was not involved in *Aglaothamnion oosumiense* (Kim et al., 1996; Kim and Kim, 1999). Although these glycoproteins must be important for fertilization, which can be blocked by adding the complimentary sugars or lectins, it is still uncertain whether these glycoproteins are responsible for species recognition. Quite recently, Kim et al. (2008) undertook a comparison of the proteome among eight isolates of the *Bostrychia radicans/moritziana* species complex showing various reproductive reactions with each other. The male and female isolates had 3.7–7.1% sex-specific proteins, and the lack of any shared sex-specific proteins across all isolates may suggest rapid evolution of these proteins (Kim et al., 2008). Such a proteome analysis is expected to become an important cue for the elucidation of the molecular mechanisms of sex as well as species recognition in red algae.

In *Chlamydomonas* gametes, sex-cell contact at fertilization is based on the complementarity between special mating-type glycoproteins (agglutinin) located on the flagellar membranes (Goodenough and Adair, 1989), and the gene sequences from these proteins are strikingly different between the closely related

species (Lee et al., 2007). Bolwell et al. (1980) reported that membrane fractions isolated from either eggs or sperms of *Fucus serratus* inhibited fertilization in a species-specific manner, and suggested that this inhibitory activity was associated with a high-molecular-weight glycoprotein containing α-fucose and α-mannose. The species-specificity of such a glycoprotein was confirmed by immunological studies (Jones et al., 1988, 1990).

By considering the fact that spermatial binding to trichogynes is not species-specific, but occurs between closely related species (Magruder, 1984), the biochemical or mechanical isolation, if present, can be presumed to work after the attachment of spermatia to trichogyne. Engel et al. (1999) evaluated the success rate of male fertilization in a natural population of *Gracilaria gracilis* by assessing the individual contribution of different males to carposporophytes, and by determining the paternity using two microsatellite loci. They found significant inter-male differences in success rate of fertilization, regardless of the distance between the male and female gametophytes, and suggested the possibility of nonrandom mating, resulting either from female choice or from male-to-male competition. This nonrandom mating was confirmed by crossing experiments using multiple individuals as sources of spermatia (Engel et al., 2002), and these results may implicate that inequality of male performance in postadhesion events generates nonrandom mating.

The behavior of spermatial nuclei after attachment of spermatia to trichogynes has been observed using time-lapse video microcopy. In Rhodomelaceae, the spermatial nucleus divides once after the attachment and the two nuclei are injected into the trichogyne, one moving down to the base and the other moving up to the tip (Pickett-Heaps and West, 1998; Wilson et al., 2002, 2003). Pickett-Heaps and West (1998) observed the attachment of multiple spermatia to a trichogyne and the movement of these nuclei within trichogynes in *Bostrychia moritziana*. They suggested that the transport systems of different gamete nuclei may interact with one another during the active transfer toward the carpogonial nucleus. Although there is still much to learn about gamete recognition and isolation mechanism before examining karyogamy in red algae, continued research, using methodologies as cited earlier may provide better understanding regarding what and how prezygotic biochemical isolating mechanisms function in red algae.

3. Postzygotic Isolating Mechanism

Although no prezygotic isolating mechanism has been established and zygotes are formed between two populations, genetic differences have become very significant, such that the resulting hybrids are less viable or less fertile than the parents. Artificial crossing experiments, usually performed among closely related species or within the same species, make it possible to reveal potential

compatibility and the degree of reproductive isolation between them. In the case of red algae, various kinds of postzygotic isolating phenomena, such as the formation of sterile/inviable F_1 gametophytes or sporophytes, abortive cystocarps (Fig. 3), or pseudocystocarps (Fig. 4), have been frequently observed using culture strains.

3.1. INVIABLE OR STERILE F_1 GAMETOPHYTES

Although production of fertile F_1 tetrasporophytes is sometimes regarded as a successful cross, it is possible that their tetraspore germlings do not grow well or grow into sterile F_1 gametophytes. Production of such inviable or sterile F_1 gametophytes is evident as the first stage of postzygotic isolation. Miura et al. (1992) performed hybridization using pigmentation variants of *Porphyra tenera* and *P. yezoensis*, which were compatible and produced fertile F_1 heterozygous sporophytes showing wild-type color. However, most of the F_1 gametophytic germlings died at the four-cell stage, and very few single-colored or chimeric gametophytes survived. Meiosis was observed to occur during conchospore germination in *P. yezoensis*, based on the evidences of chimeric blade formation (Ohme and Miura, 1988) and cytogenetic analyses (Ma and Miura, 1984; Shimizu et al., 2008; but see Wang et al., 2006 for different position of meiosis), and thus, the breakdown of F_1 gametophytes could have probably been caused by defective meiosis (Miura et al., 1992).

Kudo and Masuda (1986) demonstrated a variety of reproductive reactions between crosses of *Polysiphonia akkeshiensis* and *P. japonica* from central and northern Japan. *Polysiphonia akkeshiensis* females were intersterile with *P. japonica* males, while the reciprocal crosses frequently produced fertile F_1 tetrasporophytes. However, the tetrasporelings did not grow well in some crosses, or in other crosses, they grew into sterile gametophytes. Rueness (1973) obtained fertile F_1 tetrasporophytes in the crosses between *Polysiphonia boldii* from Texas, USA, and *P. hemisphaerica* from Scandinavia. Although more than 1,000 tetraspores were discharged from the hybrid tetrasporophytes, most of them failed to develop after a few divisions and only a few spores grew into fertile gametophytes. Although F_1 tetrasporophytes released tetraspores in most of the crossings of *Digenea simplex* from the Atlantic and Caribbean Seas, the percentage of healthy tetrasporelings were apparently higher in the crossings between the adjacent populations (77–100%) than in the crossings between the distant populations (3–43%) (Pakker et al., 1996).

Meiosis has been observed in the formation of tetraspores in some florideophycean species, such as *Wrangelia plumosa* and *Antithamnionella pacifica* (Goff and Coleman, 1990), and some parasitic species of *Janczewskia*, *Levringiella*, *Gonimophyllum* (Kugrens and West, 1972), and *Choreocolax* (Goff and Coleman, 1984). However, *Scagelia pylaisaei* has been observed to undergo

meiosis during tetraspore germination like *Porphyra* (Goff and Coleman, 1990). According to the cytological observations of *Palmaria palmata* from Atlantic Canada and Ireland, which produced inviable F_1 gametophytes, the hybrid tetrasporophytes showed complex chromosome pairings, which may explain meiotic abnormality (van der Meer, 1986, 1987). Deficient meiosis may probably be one of the reasons for inviable hybrid gametophytes, but cannot easily explain various abnormalities, from no germination of tetraspores to production of abortive cystocarps on F_1 hybrid females.

3.2. ABORTIVE CYSTOCARPS OR INVIABLE F_1 SPOROPHYTES

In contrast to most of the organisms in which a zygote is released from the gametophyte to produce a diploid individual, a red algal zygote grows into a multicellular carposporophyte and discharges diploid carpospores while attached to the female gametophyte, and hence, cystocarp development is a good indicator of reproductive affinity.

Abortive cystocarps (Fig. 3) that fail to discharge carpospores or are disrupted before sporulation, or inviable F_1 sporophytes, have also been observed in various crosses. For instance, abortive cystocarps were produced between a *Polysiphonia acuminata* male from California and a *P. japonica* female from Korea, whereas nonviable F_1 tetrasporophytes appeared in the reciprocal cross (Yoon and West, 1990). The cross between a male *Gracilaria foliifera* from the UK and a *G.* sp. female from Italy produced cystocarps that failed to discharge carpospores, while no reaction occurred in the reciprocal cross (Bird and McLachlan, 1982). Formation of abortive cystocarps or nonviable F_1 sporophytes undoubtedly reduces fitness, and such partially compatible pairs are usually found from geographically distant populations (Table 1).

Developmental patterns of the carposporophyte are diverse within the florideophycean algae and have been used as key systematic characters for generic or higher taxonomic ranks (Hommersand and Fredericq, 1990), whereas the physiological or genetic studies on this stage have not progressed. It is known that carposporophytes of some red algae discharge many carpospores for several weeks, with the total numbers of spores released reaching into the thousands (Boney, 1960; Wilce and Sears, 1991; West and McBride, 1999; Kamiya and Kawai, 2002). The number of cystocarps and discharged carpospores per cystocarp is apparently various among the cross pairs even in the same species. More cystocarps are produced and more carpospores are discharged from each cystocarp in the self-crossing than the outcrossing between the distantly distributed gametophytes (M. Kamiya and J. A. West, unpublished data, 2008). A negative correlation between the number of cystocarps and the geographical distance has been reported by Zuccarello and West (1995). Such variations of carposporophyte productivity possibly represent the intraspecific

difference of reproductive affinity, and more detailed analyses on carposporophyte development and carpospore formation are required for elucidating the genetic impact of outcrossing.

3.3. PSEUDOCYSTOCARPS

A cystocarp-like swelling, usually called pseudocystocarp (Fig. 4), is frequently observed in red algal crosses. Although this structure develops pericarps and an ostiole, there is no or little initial carposporophyte tissue formed and no carpospores are discharged. McLachlan et al. (1977) observed pseudocystocarp formation in the crosses between *Gracilaria foliifera* from the UK and *G. tikvahiae* (as *G.* sp.) from Nova Scotia. Pseudocystocarps were also produced in the cross between a *G. cervicornis* male and a *G. mammillaris* female from Brazil, but no pseudocystocarps occurred in the reciprocal cross (Plastino and Oliveira, 1988). Most of the cross pairs forming pseudocystocarps were isolated from geographically distant populations, between which genetic exchange must be quite low (Table 1).

Pseudocystocarps are formed in crosses showing various degrees of genetic diversity; the sequence divergence of the Rubisco spacer and its flanking regions is found to be 0.6–1.7% in *Bostrychia radicans* (Zuccarello and West, 1997), 0.4–2.6% in *Caloglossa leprieurii* (Kamiya et al., 1998), 3.7% in *C. postiae* (Kamiya et al., 1999), and 0.6–5.8% in *C. monosticha* (M. Kamiya and J. A. West, unpublished data, 2008). Crossability, including production of pseudocystocarps, can be considered as a symplesiomorphy, a maintained ancestral characteristic, and hence, the interfertile entities, regardless of whether fully or partially, may not necessarily be close phylogenetically (Donoghue, 1985). In fact, there are some reports of the inconsistency between crossability and genetic distance (e.g., Pakker et al., 1996; Zuccarello and West, 1997), and hence, caution must be used in assessing reproductive compatibility as a phylogenetic similarity indicator.

The mechanism causing the improper development of carposoporphyte (i.e., pseudocystocarp) is poorly known. McLachlan et al. (1977) reported that syngamy occurred in these crosses as pseudocystocarps did not form either in the presence of heat-killed spermatia or filtrates from suspensions of viable spermatia. Destombe et al. (1990) indicated that the frequency of pseudocystocarp production increased with the age of the male gametes. Boo and Lee (1983) observed the early discontinuance of carposporophyte development in a cross of *Antithamnion defectum* male × *A. sparsum* female. Pseudocystocarp formation is assumed to be under the control of interactions between maternal haploid tissue and zygotic diploid tissue (Hommersand and Fredericq, 1990). As pseudocystocarps are usually produced between morphologically similar or identical entities, the formation of such structures has been considered as

Table 1. A summary of the red algal crossing results.

Species (distribution)	Compatibility	Comments	Reference
Bangiales			
Porphyra anagusta (Tokyo, JPN), *P. pseudolinearis* (Miyagi, JPN), *P. tenera* (Fukushima, Miyagi and Tokyo, JPN), *P. umbilicalis* (Tokyo, JPN) and *P. yezoensis* (Tokyo, JPN)	F_1 gametophytes fertile in the crosses among *P. anagusta*, *P. pseudolinearis,* and *P. umbilicalis*. These species are fertile with *P. tenera* and *P. yezoensis,* but most of the F_1 gametophytes are abnormal.		Suto (1963)
Porphyra tenera (green mutant; locality unknown) and *P. yezoensis* (wildtype, green and red mutants; JPN)	F_1 sporophytes fertile, but most F_1 gametophytes become extinct at the four-cell stage in either interspecific or intermutant crosses.	F_1 gametophytes between the different color mutants were chimeric.	Miura et al. (1992)
Palmariales			
Palmaria palmata var. *palmata* (Canada and Ireland) and var. *sobolifera* (Ireland)	Viable F_1 gametophytes produced in the intervariety crossings of the Irish strains, but inviable F_1 gametophytes produced between these Irish strains and Canadian var. *palmata*.		van der Meer (1987)
Gracilariales			
Gracilaria foliifera (UK), *G. tikvahiae* (Atlantic Canada), *G.* sp.1 (Italy) and *G.* sp.2 (Pacific USA)	Fertile cystocarps failed to discharge spores between *G. foliifera* male and *G.* sp.1 female. No reaction or pseudocystocarps produced in other crosses.		McLachlan et al. (1977), Bird and McLachlan (1982)
Gracilaria multipartita (France, Ireland and UK) and *G. tikvahiae* (Pacific Canada, North Carolina and Texas in USA)	Fertile F_1 tetrasporophytes produced in the intraspecific crosses, but no reaction in the interspecific crosses	The strain from North Carolina was more similar to the European strains than the Texas strain.	Guiry and Freamhainn (1986)
Gracilaria verrucosa (now a synonym of *G. gracilis*; Pacific Canada and UK)	No reproductive reaction	The chromosome number was different between them (n = 32 for British strain and n = 24 for Canadian strain).	Bird et al. (1982)
Gracilaria verrucosa (now a synonym of *G. gracilis*; Shinori and Kikonai, JPN) and *G. vermiculophylla* (Akkeshi, JPN)	F_1 gametophytes fertile in all the crosses.		Yamamoto and Sasaki (1987, 1988)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Gracilaria verrucosa (now a synonym of *G. gracilis*; Argentina, Pacific Canada, France, Norway and UK)	Viable or inviable F_1 tetrasporophytes produced among the strains from Argentina, France, Norway, and UK. The strain from Pacific Canada interster-ile with that from UK.		Rice and Bird (1990)
Gracilaria verrucosa (now a synonym of *G. gracilis*; five sites in France)	Both normal and abortive cystocarps produced in the most crosses attempted.	Fertility was significantly different among the females, but not different among the males.	Richerd et al. (1993)
Gracilaria cervicornis (Brazil and Pacific USA), *G. mammillaris* (Brazil) and *G.* aff. *verrucosa* (now a synonym of *G. gracilis*; Brazil and Pacific USA)	Normal cyctocarps produced in Brazilian male × US female of *G. cervicornis* but no reaction in the reciprocal cross. Normal cyctocarps produced between the strains of *G.* aff. *verrucosa*. Pseudocystocarps produced in the Brazilian male of *G. cervicornis* × *G. mammillaris* female, but no reaction in the reciprocal cross.		Plastino and Oliveira (1988)
Gracilaria tikvahiae (Canada and Florida)	Incomplete.		van der Meer (1986)
Gelidiales			
Gelidium pulchellum (France and Ireland), *G. pusillum* (France, Ireland, Norway and UK)	Fertile F_1 tetrasporophytes produced in the intraspecific crosses, but no reaction in the interspecific crosses.		Fredriksen et al. (1994)
Gigartinales			
Gigartina teedii (now a synonym of *Chondracanthus teedei*; Brazil, France, Greece, Ireland, Sicily and UK)	F_1 tetrasporophytes fertile.	Growth rate was different between the Mediterranean and Atlantic strains. Gross morphology and color were different among these strains.	Guiry (1984), Guiry et al. (1987)
Petrocelis middendorfii (Alaska) and *P. franciscana* (now a synonym of *Mastocarpus papillatus*; two sites in California)	Carpospores produced.		Polanshek and West (1975)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Male of *Gigartina papillata* (now a synonym of *Mastocarpus papillatus*; seven sites in California) and female of *Petrocelis middendorfii* (eight sites in California)	Carpospores produced in some crosses, but not in other crosses.		Polanshek and West (1977)
Gigartina pacifica (now a synonym of *Mastocarpus pacificus*; four sites in Hokkaido, JPN)	F_1 sporophytes produced in the most crosses.		Masuda et al. (1984)
Ahnfeltiopsis concinna (Hawaii and six sites in JPN)	Fertile F_1 gametophytes produced.	No morphological difference among them.	Masuda and Kogame (1998)
Mastocarpus sp. (now regarded as *M. yendoi*; eight sites in JPN)	Fertile F_1 sporophytes produced in the most crosses.		Masuda et al. (1987)
Gigartina stellata and *Petrocelis cruenta* (now synonyms of *Mastocarpus stellatus*; France, Iceland, Ireland, Portugal, Spain and UK)	Carpospores released among the northern populations or among the southern populations, but no reaction between them.	The northern and southern populations were morphologically distinguishable.	Guiry and West (1983)
Gigartina agardhii (now a synonym of *Mastocarpus jardinii*; seven sites in California)	Carpospores produced and germinated in the most crosses attempted.		West et al. (1978)
Chondrus crispus (Atlantic Canada, France, Germany, Iceland, Norway, two sites in Spain, five sites in Ireland, and four sites in UK)	Carpospores released in all the crosses attempted, and fertile tetrasporophytes produced in 10% of the crosses		Guiry (1992)
Chondrus crispus (Bay of Fundy and Gulf of St. Lawrence, Nova Scotia, Canada)	Cystocarps produced on the female from Gulf of St. Lawrence with the male from Bay of Fundy, but no reaction in the reciprocal cross.	The gross morphology and developmental response to temperature were different between the two strains.	Chen and Taylor (1980)
Chondrus pinnulatus f. *pinnulatus* (Abashiri, Hanasaki, Muroran and Oshoro, JPN), *C. pinnulatus* f. *armatus* (now *C. armatus*; Fukaura, Kikonai, Nemuro, Oma and Oshoro, JPN)	F_1 gametophytes fertile in the intraformae crosses. Results of interformae crosses variable from no reaction to formation of fertile F_1 gametophytes.	Upper temperature tolerance was different between the two formae.	Brodie et al. (1997)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Chondrus nipponicus (two sites in JPN), *C. ocellatus* f. *crispoides* (Aomori, JPN) and f. *ocellatus* (China, Korea and three sites in JPN)	Fertile F_1 tetrasporophytes produced in the intraformae crosses. Results of interformae crosses were variable from no reaction to formation of viable F_1 gametophytes. Fertile F_1 tetrasporophytes produced between *C. nipponicus* and f. *crispoides*.	In f. *ocellatus*, the strains from China, Korea, and one Japanese site required long-day condition for gametogenesis, whereas the other two Japanese strains required short-day condition.	Brodie et al. (1993)
Digenea simplex (Atlantic USA, Netherlands Antilles, Cape Verde Islands and Western Australia)	Various percentages of healthy tetrasporelings produced in the crossings, except for the Western Australian strain, which produced sterile F_1 sporophytes with the strains from Atlantic USA and Cape Verde Islands, but showed no reaction with other strains.	Despite the reduced level of sexual compatibility between Caribbean and Cape Verde Islands isolates, they shared position in the RAPD analysis and showed similar temperature responses.	Pakker et al. (1996)
Ceramiales			
Aglaothamnion byssoides (Sweden and Atlantic USA) and *A. furcellariae* (France) (now synonyms of *A. tenuissimum*)	The Swedish *A. byssoides* intersterile with the American *A. byssoides,* but produced F_1 tetrasporophytes with *A. furcellariae*.	These interfertile strains were morphologically similar to each other.	L'Hardy-Halos and Rueness (1990)
Aglaothamnion byssoides (now a synonym of *A. tenuissimum*; *Sweden) and A. tenuissimum* var. *mazoyerae* (Italy)	F_1 tetrasporophytes produced in the interspecific crosses.		Furnari et al. (1998)
Antithamnion defectum (Pacific USA) and *A. sparsum* (two sites in Korea)	Viable carpospores released between *A. sparsum* male and *A. defectum* female, but no carpospores released in the reciprocal cross.		Boo and Lee (1983)
Antithamnion plumula var. *bebbii* (now a synonym of *Pterothamnion crispum*; three sites in Norway and Sweden), var. *crispum* (now a synonym of *P. crispum*; two sites in UK) and var. *plumura* (four sites in Norway and UK)	Gonimoblasts developed in the intravariety crosses and between all strains of var. *bebbii* and var. *plumura* from Hoftoy in Norway and Plymouth in UK. No reaction in other intervariety crosses.	Interfertile strains of var. *bebbii* and var. *plumura* were isolated from adjacent sites in Norway.	Sundene (1975)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Antithamnion plumula var. *bebbii* (now a synonym of *Pterothamnion crispum*; Sweden) and var. *plumura* (UK)	F_2 gametophytes fertile in the intervariety crosses.	The heterozygous tetrasporophytes always showed the var. *plumula* phenotype and their tetraspores grew into the two varieties in equal numbers.	Rueness and Rueness (1975)
Callithamnion byssoides (now a synonym of *Aglaothamnion tenuissimum*; Norway and four Atlantic sites in USA) and *C. halliae* (now a synonym of *A. halliae*; Texas, USA)	*Callithamnion halliae* fertile with the four American strains but sterile with the Norwegian strain of *C. byssoides*. The four American strains were interfertile with each other, but sterile with the Norwegian strain.	Thallus color was different between *C. byssoides* and *C. halliae* but they were morphologically indistinguishable. Their hybrids showed intermediate color.	Spencer et al. (1981)
Callithamnion boergesenii (now a synonym of *Aglaothamnion boergesenii*; Puerto Rico) and *C. byssoides* (now a synonym of *A. tenuissimum*; North Carolina and Georgia, USA)	The two American strains of *C. byssoides* interfertile with each other, but sterile with *C. boergesenii*.		Aponte and Ballantine (1990)
Ceramium strictum (now a synonym of *C. tenuicorne*; Norway and Atlantic USA) and *C. tenuicorne* (Baltic Sea)	The US *C. strictum* intersterile with other strains and F_1 gametophytes produced between the Norwegian *C. strictum* and *C. tenuicorne*.	The morphology and growth response against salinity regime were similar between the US and Norwegian *C. strictum,* but obviously different between *C. strictum* and *C. tenuicorne*.	Rueness (1978)
Ceramium strictum (now a synonym of *C. tenuicorne*; Denmark, Norway and Sweden)	F_1 gametophytes fertile.	Salinity tolerance was different among the strains and their hybrids showed intermediate responses between the parents.	Rueness and Kornfeldt (1992)
Ceramium tenuicorne (three strains from Skagerrak and six strains from Baltic Sea)	Most intraspecific crosses attempted successful, resulting in germinating carpospores.		Gabrielsen et al. (2003)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Ceramium aff. *rubrum* (now a synonym of *C. virgatum*; each two sites in Washington, USA and Nova Scotia, Canada)	F_1 gametophytes fertile between one American strain and one Canadian strain, but no cystocarps or no viable carpospores produced in the other crosses.		Garbary (1988)
Caloglossa continua ssp. *axillaris* (now a synonym of *C. monosticha*; north Australia), ssp. *continua* (JPN, Taiwan and Vietnam), *C. monosticha* (Indonesia, Singapore and west Australia) and *C. saigonensis* (Malaysia)	Fertile F_1 gametophytes produced within ssp. *continua* and between the Australian and Indonesian *C. monosticha*. Variable reaction observed in the most other crosses, from no reaction to production of inviable F_1 gametophytes.		Kamiya et al. (2003)
Caloglossa intermedia (Georgia and South Carolina, USA)	Fertile F_1 gametophytes produced.		Kamiya et al. (2000)
Caloglossa leprieurii (South Africa, Venezuela, Peru, Atlantic USA and each two sites in JPN and Singapore)	Five mating groups recognized, and no reaction or only pseudocystocarps observed between the different mating groups.	One mating group was morphologically distinguishable from the others.	Kamiya et al. (1998), Kamiya (2004)
Caloglossa leprieurii (now regarded as *C. vieillardii*; Fiji, three sites in JPN and four sites in Australia)	Four mating groups recognized. Inviable F_1 gametophytes or sporophytes produced between the Japanese and Western Australian mating groups, while no reaction or only pseudocystocarps observed between the other mating groups.		Kamiya et al. (1995), Kamiya (2004)
Caloglossa monosticha (six sites in Australia and three sites in Indonesia)	Pseudocystocarps, inviable F_1 sporophytes or inviable F_1 gametophytes produced between the two Australian strains and the other strains. Fertile F_1 gametophytes produced in the most other crosses.		Kamiya and West (2008)
Caloglossa postiae (Australia and two sites in JPN)	Pseudocystocarps produced between the Australian and Japanese strains. Fertile F_1 gametophytes produced between the Japanese strains.		Kamiya et al. (1999)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Bostrychia radicans (seven sites in Atlantic North America and seven sites in Pacific North America)	Viable F_1 sporophytes produced among the Pacific populations. At least six mating groups recognized along the Atlantic coast, and pseudocystocarps were produced between some mating groups. Inviable F_1 gametophytes produced in the cross between South Carolina and Pacific Mexico.	There was a negative correlation between the number of cystocarps and geographical distance.	Zuccarello and West (1995)
Bostrychia moritziana (New Zealand, Fiji, Indonesia, and several sites in Australia and South Africa)	The Indonesian strain sterile with any other strains. The South African strain displayed a lower compatibility with those from Australia, New Zealand and Fiji, which produced viable F_1 sporophytes with each other.		Zuccarello et al. (1999)
Bostrychia moritziana (Australia and South Africa) and *B. radicans* (Australia, Pacific Mexico, Peru, Brazil, Venezuela and Atlantic USA)	Carpospores released in the intraspecific cross of *B. moritziana*, and at least four mating groups recognized in *B. radicans*. No reaction observed between the different mating groups or in the interspecific crosses.		Zuccarello and West (1997, 2003)
Laurencia japonensis (three sites in JPN), *L. nipponica* (three sites in JPN) and *L. okamurae* (three sites in JPN)	Fertile F_1 gametophytes produced in all the intraspecific crosses. Only pericarps developed in some crosses between the *L. okamurae* male and *L. nipponica* female, while no reaction observed in the other interspecific crosses.		Abe and Masuda (1998)
Murrayella periclados (Guam, French Polynesia, Fiji, Indonesia, Mindanao and Cebu in Philippines)	Carpospores germinated in most crosses, but failed to germinate between the male from French Polynesia, Cebu or Indonesia and the female from Mindanao or Guam.		Zuccarello et al. (2002)
Polysiphonia ferulacea (now a synonym of *Neosiphonia ferulacea*; North Carolina, USA and Bermuda, UK)	No response observed between the two strains.	Chromosome number was different between the two strains.	Kapraun (1977)

(continued)

Table 1. (continued)

Species (distribution)	Compatibility	Comments	Reference
Polysiphonia acuminata (Pacific USA) and *P. japonica* (Korea)	Abortive cystocarps produced between *P. acuminata* male and *P. japonica* female and F_1 tetrasporophytes inviable in the reciprocal cross.		Yoon and West (1990)
Polysiphonia akkeshiensis (three sites in JPN) and *P. japonica* (five sites in JPN) (now regarded as *Neoshiphonia harveyi*)	F_2 tetrasporophytes fertile in the intraspecific crosses. Results of the interformae crosses variable from no reaction to formation of fertile F_2 tetrasporophytes.	The morphological difference between the two species was not maintained in the culture conditions.	Kudo and Masuda (1986)
Polysiphonia boldii (Texas, USA) and *P. hemisphaerica* (now a synonym of *P. boldii*; Scandinavia)	Most F_1 gametophytes inviable.		Rueness (1973)
Spyridia filamentosa (South Australia, Philippines, Pacific Mexico and Puerto Rico)	Carpospores released in the cross between the Australian male and Mexican female, while no reaction or only pseudocystocarps produced in other crosses.		Zuccarello et al. (2002)

indicative of an evolutionary link between them (Guiry et al., 1987). In the absence of spermatia, formation of pseudocystocarps has been observed occasionally in *Bostrychia* (J. A. West, unpublished data, 2008), *Caloglossa* (Tanaka and Kamiya, 1993), and *Gracilaria* (Bird and McLachlan, 1982). Zuccarello et al. (2002) tried intraspecific hybridization among worldwide populations of *Spyridia filamentosa* and *Murrayella periclados*, and pseudocystocarps were produced not only in unsuccessful crosses, but also in successful crosses that produced normal cystocarps.

4. Relationships Between Reproductive Affinities, Geographic Distance, and Genetic Diversity

Positive compatibility has been observed in many crossing experiments between geographically distant populations, and in some cases, their genetic similarities were also confirmed by DNA markers. For example, interfertility was demonstrated in *Chondrus crispus* from the east and west Atlantic coasts (Guiry, 1981, 1992). In the molecular phylogenetic analyses of *C. crispus* from Europe and Pacific Canada

based on the ITS1 sequence data, the genetic distance was not to be correlated with the geographic distance at all, and hence, multiple transatlantic dispersal was suggested (Hu et al., 2007). Interfertility between geographically distant populations was also demonstrated in *Bostrychia radicans* from both sides of South America (Pacific Mexico vs. Venezuela, or Peru vs. Brazil), and there were only a few site changes at the Rubisco spacer plus the flanking genetic region among these interfertile strains (Zuccarello and West, 1997). Furthermore, the Pacific Mexican strains were more reproductively compatible as well as genealogically closer to some strains from the Atlantic USA than other U.S. strains (Zuccarello and West, 2003). They also demonstrated that the samples with the same plastid haplotypes were sexually compatible and that those with different plastid haplotypes were reproductively isolated, though there were some exceptions.

Although seaweeds are generally considered poor dispersers owing to short life of spores and gametes (Destombe et al., 1990; Santelices, 1990; Shanks et al., 2003), various seaweeds may get a lift on substrates transported long distances (van den Hoek, 1987), such as drifting *Sargassum* thalli, frequently carrying many epiphytic algae (Oliveira et al., 1979). Some red algae, such as *Bostrychia, Caloglossa,* and *Catenella*, which are abundant in pantropical estuaries, are often epiphytes on mangrove pneumatophores or trunks (Tanaka and Chihara, 1987), and hence, driftwood of mangroves may facilitate distribution of these algae. Man is undoubtedly the most potent vector of long-distance dispersal, and many algal dispersals are presumed to have been assisted by transplanted oysters, ships' hulls and ballast water, fishing nets, and plastic debris, or other unknown factors (Doty, 1961; Loosanoff, 1975; Critchley and Dijkema, 1984; Carlton and Scanlon, 1985; Hay, 1990; Barnes, 2002; Flagella et al., 2007; Zuccarello et al., 2008).

However, partial incompatibility, including production of inviable progenies and pseudocystocarps, has been mostly observed between geographically distant populations (Table 1). Variable percentages of healthy tetrasporelings were observed in the crossings of *Digenea simplex* from Caribbean coasts, mid-Atlantic Islands, and Western Australia (Pakker et al., 1996). Despite the reduced production of healthy tetrasporelings between the Caribbean and mid-Atlantic isolates, they indicated close genetic distances based on RAPD analysis and similar temperature tolerances, and these data suggest trans-Atlantic dispersal of this species in the recent geological past (Pakker et al., 1996). So far, ten mating groups have been recognized in *Caloglossa leprieurii* complex, and these mating groups are fully intersterile or partially compatible (Kamiya, 2004). In this species, without exception, reproductive isolation is completely established between the sympatrically distributed mating groups, and inviable progenies or pseudocystocarps are produced between the geographically separated groups, and never between the sympatric groups. It is evident that allopatric speciation has a great effect on the evolution of these algae like many terrestrial organisms, and that genetic exchange is surely restricted between the populations in which reproductive isolation is not fully established.

In contrast to many instances that demonstrate partial compatibility between geographically separated populations, we have little data to suggest partial compatibility between adjacent populations. One good example is indicated by Brodie et al. (1997), who performed cross hybridizations between two formae of *Chondrus pinnulatus*, f. *armatus* (described as a distinct species, *C. armatus*, in this paper), and f. *pinnulatus*, whose gross morphology was different even in the same culture condition. Forma *pinnulatus* was observed to have a more northerly distribution than f. *armatus*, but they were found to be sympatrically distributed in northern Japan. The results of interformae crosses were different, from no reproductive reaction to formation of fertile F_1 gametophytes. Their reproductive compatibilities were not correlated to their geographic distances, because f. *pinnulatus* female from Muroran was found to be interfertile with f. *armatus* male from every locality examined, and also fertile F_1 tetrasporophytes were produced between these formae isolated from the same locality. The two formae, however, showed a difference in the upper temperature tolerance, which may probably be associated with their different distribution pattern, but the gametophytes of either formae indicated similar reproductive phenology (Brodie et al., 1997). By considering their distinct morphological differences, a prezygotic isolation mechanism such as a subtle difference in maturation timing can be established. Alternatively, gene flow between the adjacent populations may be restricted even without any obvious geographic barrier to dispersal. Destombe et al. (1990) showed that the mean fertile life of spermatium in *Gracilaria gracilis* is about only five hours and that spermatial dispersal is leptokurtic and limited to less than 100 m in the field. Recent studies using molecular markers also indicate that red algal populations are highly differentiated at the level of a few to tens of kilometers (e.g., Intasuwan et al., 1993; Wright et al., 2000; Zuccarello et al., 2001; Zuccarello and West, 2003; Engel et al., 2004).

5. Perspective

Genetic variation can accumulate between reproductively compatible entities owing to external barriers or geographical separation, and the genetic distance is mostly, but not necessarily, correlated to the reproductive affinity. Therefore, it is important to couple the genetic data with information on reproductive compatibility to infer how populations differentiate, and hence, speciate. Frequent occurrence of intraspecific reproductive isolation has been revealed by crossing experiments with various species, and this information may give clues to elucidate the process of red algal speciation. Furthermore, as shown in the subsequent paragraphs, crossing studies may be indispensable for new lines of investigations on the genetic mechanisms of red algal life history variation, phenotypic variation, and adaptive evolution.

5.1. SPECIATION PATTERNS

Allopatric speciation is well documented in various organisms, whereas sympatric speciation or divergence of species without geographic isolation has become increasingly accepted as a result of theoretical works (Via, 2001). Nonetheless, very few empirical studies for sympatric speciation have been documented in nature (Schliewen et al., 1994; Filchak et al., 2000; Barluenga et al., 2006; Savolainen et al., 2006). It is still difficult to prove that speciation in a given pair of taxa has occurred in an exclusively sympatric manner, because persuasive cases of sympatric speciation must engage biogeographic and phylogenetic tracks that make the existence of an allopatric phase highly likely (Jiggins, 2006). In red algae, incompletely compatible entities were usually isolated from geographically distant populations, occasionally from adjacent populations, and this suggests that allopatric speciation is dominant in these organisms. We still do not have direct evidence to prove the incidence of sympatric speciation, and remain ignorant of how frequent this process is in red algae; hence, analyses of reproductive compatibility should be continued to unravel the pattern of red algal speciation.

5.2. APOMIXIS

Apomixis, or reproduction without fertilization and meiosis, has been observed in various red algal taxa, and spore recycling has been found to be relatively common in red algae, reported from nearly 40 genera, though parthenogenesis of gametes has been found in only a few genera (reviewed by Hawkes, 1990; West et al., 2001). In ferns and flowering plants, apomixis and polyploidization are the outcomes of the temporal deregulation of normal sexual reproductive pathways, sometimes caused by interspecific hybridization (Praekelt and Scott, 2001; Park and Kato, 2003; Schranz et al., 2005). Although red algal apomixis has been recognized for a long time, and that apomictic entities are much more dominant than sexual ones in some species and localities (Maggs, 1988), the causes of apomixes have remained unknown. Quite recently, apomictic tetrasporophytes of *Caloglossa monosticha* were unexpectedly obtained through outcrossings between a male strain from Australia and several female strains from Indonesia (Kamiya and West, 2008). As no reproductive reaction or formation of pseudocystocarps were observed in the reciprocal crosses, reproductive isolation seems to have progressed substantially between the strains. As many apomictic strains of other *Caloglossa* species have been isolated worldwide (West et al., 1994, 2001) and some of them were highly heterozygous in the nuclear actin gene (M. Kamiya and J. A. West, unpublished data, 2010), we presume that some parts of apomictic species originated from such outcrossings between genetically different entities. Further crossing experiments are required to investigate the origin of these apomicts and clarify the genetic mechanism of apomixis.

5.3. HETEROSIS

Heterosis or hybrid vigor is a phenomenon in which an F_1 hybrid has superior performance over its parents. It has been observed in many plant species (Birchler et al., 2003), and the utilization of heterosis is responsible for the commercial success of plant breeding in several crops and horticultural species (Duvick, 1999). However, there have been only a few instances suggesting macroalgal heterosis (Hara and Akiyama, 1985; Patwary and van der Meer, 1994). For example, the hybrid tetrasporophytes of *Gelidium vagum* consistently exhibited growth superiority over the inbred tetrasporophytes in all growth experiments, although the number of tetrasporangial stichidia in the hybrids were fewer than in the related inbred lines (Patwary and van der Meer, 1994). Though the discovery of heterotic hybrids and demonstration of heterosis usually requires considerable works, it is important to investigate this phenomenon, because heterosis can be exploited for developing economic seaweeds more appropriate to mariculture (see Guillemin et al., 2008). In addition, heterosis may possibly be associated with diploid dominance among populations, which have been reported in various red algae (see Fierst et al., 2005). Diploid dominance may be attributed to the enhanced fitness of the diploid phase, probably as a result of heterosis and/or the masking of deleterious recessive alleles (Guillemin et al., 2008). Heterosis is found to disappear in the next haploid gametophyte, but can be maintained through apomixis (Bilinski et al., 1989).

5.4 GENETIC BASIS OF ADAPTATION

Outcrossing experiments are sometimes indispensable for genetic analyses of morphological variation and/or physiological adaptation. As most of the phenotypic traits of interest vary in degree and can be attributed to the interactions between many genes, such polygenic traits do not follow patterns of Mendelian inheritance. Elucidating the entire complement of genes related to a polygenic trait provides the basis of understanding the effect of the genotype of an individual in nature. Chromosomal location of quantitative trait loci (QTLs) can be inferred by analyzing the band patterns of anonymous molecular markers, such as AFLP and microsatellites, on the recombinant inbred lines derived from the hybrids between the parents that show a different phenotypic trait of interest, and finding association between markers and quantitative trait. Such a QTL mapping analysis has been used for studying the genetic basis of adaptation and can provide clues about the evolutionary history of populations, causes of the population differentiation, and genetic basis of heterosis, which is still uncertain even in plants and animals (Zeng, 2005; Garcia et al., 2008). Although we do not know any QTL mapping studies on macroalgae, some of the outcrossing data introduced in this chapter should be applicable to this analysis. For example, Gabrielsen et al. (2002) found in *Ceramium tenuicorne* that the frequencies of some RAPD bands were correlated

to the salinity regime in their habitats. If these randomly derived markers are closely linked to the genes that are responsible for the adaptation to different levels of salinity, they may be good candidates for studying the molecular basis of salinity tolerance.

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TAXONOMIC REVISIONS OF FRESHWATER RHODOPHYTA IN RECENT YEARS

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1. Order Compsopogonales (Skuja, 1939). Type Family: Family Compsopogonaceae (Schmitz)

The Compsopogonales includes three genera, *Boldia, Compsopogon*, and *Pulvinaster* (Kumano, 2002; Sherwood, 2006; West et al., 2007a, b). The Compsopogonales, including three families, Boldiaceae, Compsopogonaceae, and Erythrotrichiaceae, was supported as a valid entity through the phylogenetic analysis of the *rbc*L gene and 18S rRNA gene sequences (Rintoul et al., 1998, 1999).

Among the family Compsopogonaceae, the method of cortex formation, which has been proposed as a character to distinguish the genus *Compsopogon* from the genus *Compsopogonopsis* (Krishnamurthy, 1962; Vis et al., 1992; Seto and Kumano, 1993) is not supported by analyses of molecular data. It is therefore proposed that the type genus species *Compsopogonopsis leptoclados* be placed in synonymy with *Compsopogon coeruleus*, and that family Compsopogonaceae comprises a single genus *Compsopogon* (Rintoul et al., 1998, 1999). Recently, a new genus *Pulvinaster* was described from Vanuatu as follows (West et al., 2007a, b).

1.1. GENUS PULVINASTER (WEST ET AL., 2007A, B). TYPE SPECIES: *PULVINUS VENETUS*

A cushion-shaped *Pulvinaster venetus* was described from Vanuatu (West et al., 2007a, b). Molecular analysis (SSU and *psb*A data.) placed this species in the order Compsopogonales.

The ultrastructure of genus *Pulvinus* is very similar to that of the genus *Compsopogon* and genus *Boldia*, in that the *cis*-region of Golgi bodies is not associated with a mitochondrion and the plastid has peripheral thylakoids. No pit connections occur between derivative cells.

2. Order Bangiales (Schmitz in Engler, 1892). Type Family: Family Bangiaceae Engler

Garbary et al. (1980) stated that the order Bangiales comprises a single family Bangiaceae with the genera *Porphyra* and *Bangia*. *Porphyra* is composed of strictly marine taxa, while *Bangia* is composed of marine taxa and one freshwater taxon. Additional new marine genera in the Bangiales, *Minerva* (Nelson et al., 2005), *Dione* (Nelson et al., 2005), and *Pseudobangia* (Müller et al., 2005) and a new freshwater genus *Bangiadulcis* (Nelson, 2008) have been proposed. This review examines only the freshwater taxon.

2.1. GENUS BANGIADULCIS (NELSON, 2008). TYPE SPECIES: BANGIADULCIS ATROPURPUREA ROTH (NELSON)

Geesink (1973) concluded that a freshwater *Bangia atropurpurea* and a marine *Bangia fuscopurpurea* were conspecific and should be synomymized under the older epithet *Bangia atropurpurea.*

However, the analyses of RuBisCo spacer, *rbc*L gene, and 18S rRNA gene sequences showed that the North American and European freshwater specimens are nearly identical and appear to be considerably different from marine specimens (Müller et al., 1998a, b).

Sequence analyses reveal a strong similarity between *Bangia* isolates in eastern Australia over a wide geographical range, but the taxonomy of *Bangia atropurpurea* may need to be re-examined in light of sequence differences between these and northern hemisphere isolates of this species (Woolcott and King, 1998; Müller et al., 2003; Niwa et al., 2003; Hanyuda et al., 2004). A new monotypic genus *Bangiadulcis* has been described to accommodate this species, *Bangiadulcis atropurpurea* (Nelson, 2008).

3. Order Acrochaetiales (Feldmann, 1953). Type Family: Family Acrochaetiaceae (Fritsch)

The genus *Balbiania* was treated as a synonym of the genus *Audouinella* and the freshwater species were transferred from the genus *Acrochaetium* to *Audouinella* (Garbary, 1987). However, *Balbiania* is recognized as a valid taxon (see Order Balbianiales). Skuja (1934) proposed criteria to distinguish the freshwater *Audouinella* from chantransia phases of *Batrachospermum*: true species of *Audouinella* having a reddish color, with chantransia-phases of *Batrachospermum* having a bluish color.

A number of fields collected or cultured bluish specimens either produced or could be induced to produce juvenile *Batrachospermum* gametophytes (Necchi and Zucchi, 1997). The morphological and molecular data strongly support that *Audouinella macrospora* represents a single species, *Batrachospermum macrosporum* and specimens of *Chantransia pygmaea* were linked to *Batrachospermum* section *Contorta* and one was unequivocally *Batrachospermum ambiguum*

(Chiasson et al., 2007). The distinctive morphology of *Ptilothamnion richardsii* was observed in two divergent clades within the Batrachospermales, associated with *Batrachospermum antipodites* and within *Batrachospermum* section *Contorta* (exact species could not be determined); therefore, the form name *Chantransia richardsii* was proposed to designate specimens with *Ptilothamnion*-like morphology, a basionym *Ptilothamnion richardsii* (Vis et al., 2006). Consequently, it has been proposed to maintain *Chantransia pygmaea*, *C. macrospora*, and *C. richardsii* (formerly *Ptilothamnion richardsii*, Ceraliales) as form taxa (Vis et al., 2006). With the addition of data (Chiasson et al., 2007), the form taxon *Chantransia pygmaea* is now unequivocally associated with five taxa, *Batrachospermum ambiguum, B. arcuatum, B. atrum, Nemalionopsis tortuosa*, and *Thorea violacea*, through sequence data and culture studies.

4. Order Balbianiales (Sheath and Müller, 1999). Type Family: Family Balbianiaceae (Sheath and Müller)

A single family is recognized, family Balbianiaceae consisting of two genera, *Balbiania* and *Rhododraparnaldia*.

4.1. GENUS BALBIANIA (SIRODOT, 1876: 149). TYPE SPECIES: BALBIANIA INVESTIENS SIRODOT

The genus *Balbiania* was treated as a synonym of *Audouinella* (Garbary, 1987). However, *Balbiania* was separated from *Audouinella* and *Rhododraparnaldia* (Sheath and Müller, 1998). Although the genus *Balbiania* and the genus *Rhododraparnaldia* are grouped together and this lineage warrants ordinal status among neighboring lineages, the phylogenetic affinities of this new order relative to higher-level lineages discussed herein remain equivocal (Harper and Saunders, 1978). Based on the analyses in terms of ultrastructure and molecular data; the *rbc*L gene, 18S rRNA gene as well as the first internal transcribe spacer region (ITS1) of the RNA genes, it is clear that the genus *Balbiania* is a valid taxon and that it is phylogenetically associated with genus *Rhododraparnaldia.* Order Balbianiales was proposed (Sheath and Müller, 1999).

4.2. GENUS RHODODRAPARNALDIA (SHEATH ET AL., 1994). TYPE SPECIES: RHODODRAPARNALDIA OREGONICA (SHEATH ET AL., 1994)

Since *Rhododraparnaldia oregonica* has characteristics of both the orders Acrochaetiales and Batrachospermales, its taxonomic status was uncertain (Sheath et al., 1994). Using *rbc*L gene and 18S rRNA gene sequences, *Rhododraparnaldia oregonica* consistently occurs on an early branch within the Acrochaetiales–Palmariales clade and does not appear to be a member of the Batrachospermales (Vis et al., 1998).

5. Order Batrachospermales (Pueschel and Cole, 1982). Type Family: Family Batrachospermaceae (C. Agardh)

The order Batrachospermales formerly comprised four families: Batrachospermaceae, Psilosiphonaceae, Lemaneaceae, and Thoreaceae. However, the family Thoreaceae was raised to ordinal status order Thoreales, with two recognized genera, *Thorea* and *Nemalionopsis*, proposed (Müller et al., 2002). In addition, a new genus *Balliopsis* was established and left as a genus *incertae sedis* within the Batrachospermales (Saunders and Necchi, 2002). Recently, the genus *Petrohua,* with morphology similar to the genera *Lemanea* and *Paralemanea*, was not only established but also not placed in a family (Vis et al., 2007). The Batrachospermales currently includes three families, Batrachospermaceae, Psilosiphonaceae, and Lemaneaceae, having *Lemanea*-type life history and pit connections with two pit plug cap layers and an enlarged outer layer.

5.1. FAMILY BATRACHOSPERMACEAE (AGARDH, 1824). TYPE: GENUS *BATRACHOSPERMUM* ROTH

In the Batrachospermaceae, four genera, *Batrachospermum, Sirodotia, Tuomeya*, and *Nothocladus*, are currently recognized at the generic level.

5.1.1. Genus Batrachospermum (Roth, 1797). Type Species: Batrachospermum gelatinosum (Linnaeus) (De Candolle, 1801)

Genus *Batrachospermum* consists of two subgenera, *Acarposporophytum* that has a carposporophyte reduced to a one-celled zygote and *Batrachospermum* (Necchi, 1987).

5.1.1.1. Subgenus Batrachospermum (Necchi, 1987). Type Species: *Batrachospermum gelatinosum* (Linnaeus) (De Candolle, 1801)

In the subgenus *Batrachospermum,* eight sections, namely, *Batrachospermum*, *Virescentia, Setacea, Turfosa, Gonimopropagulum, Hybrida, Aristatae*, and *Contorta*, have been recognized. However, the section *Hybrida* has been subsumed into section *Contorta*. Based on *rbc*L and 18S rRNA gene sequences, subgenus *Batrachospermum* appears to comprise many morphologically similar but distantly related taxa, which will need further investigation to resolve their taxonomic status at sectional level (Vis et al., 1998).

5.1.1.2. Section Batrachospermum. Type Species: *Batrachospermum gelatinosum* (Linnaeus) (De Candolle, 1801)

The fact that the type species of genus *Batrachospermum* (*B. gelatinosum*) is included within a strongly supported clade with family Lemaneaceae, genus *Sirodotia* and genus *Tuomeya* makes this section a very difficult nomenclatural entity (Vis and Entwisle, 2000).

5.1.1.3. Section Setacea (De Toni, 1897). Type: *Batrachospermum dillenii* (Bory)

B. atrum (section *Setacea*) and *B. helminthosum* (section *Virescentia*) are on divergent branches based on sequence data for 18S rRNA and the *rbc*L (Vis et al., 1998), and Kumano (2002) also prefers to keep both sections separated. This result supports the hypothesis that the section *Setacea* is not made up of merely small whorl members of the section *Virescentia* (Necchi and Entwisle, 1990).

5.1.1.4. Section Turfosa Sirodot Emmend. Necchi (1990). Type Species: *Batrachospermum turfosum* (Bory)

5.1.1.5. Section Virescentia (Sirodot, 1873). Type Species: *Batrachospermum coerulescens* (Sirodot)

The placement of *B. turfosum* (section *Turfosa*) and *B. helminthosum* (section *Virescentia*) differs between analyses, but the two taxa are within the large mixed *Batrachospermum* clade (Vis and Entwisle, 2000).

5.1.1.6. Section Gonimopropagulum (Sheath and Whittick, 1995). Type: *Batrachospermum breutelii* (Rabenhorst)

Sheath and Whittick (1995) established this section, which is named to denote the zonately divided propagules formed by gonimoblasts.

5.1.1.7. Section Aristata (Skuja, 1933). Type: *Batrachospermum cayennense* (Montagne)

Kumano (1993) divided section *Aristata* into subsection *Aristata* containing *B. caynennse* and subsection *Macrospora* containing *B. macrosporum,* which appears to fit with molecular data showing that these taxa are not closely related (Vis et al., 1998; Vis and Entwisle, 2000). These subsections may be raised to section level after more molecular data are obtained (Vis et al., 2005).

5.1.1.8. Section Contorta Skuja Emended (Vis and Entwisle, 2000). *Heterotypic Synonym*: Section Hybrida Sirodot ex De Toni. Type Species: *Batrachospermum procarpum* (Skuja)

Taxa from the section *Contorta* have formed a well-supported clade (Vis et al., 1998; Vis and Entwisle, 2000). With the section *Hybrida* subsumed into section *Contorta*, no subsectional classification has been proposed (Vis and Entwisle, 2000). Section *Contorta* is well supported with the addition of sequences from five French Guiana species. However, the subsectional classification (Kumano, 1982, 2002) was not supported by molecular data (Vis et al., 2005).

5.1.2. Genus Sirodotia (Kylin, 1912). Synonym: Section Sirodotia (Kylin) Necchi and Entwisle. Type Species: Sirodotia suecica (Kylin)

Genus *Sirodotia* established by Kylin (1912) was distinguished from other related taxa based on characteristics of asymmetric trichogyne and defused carposporophytes

(Kumano, 1982; Sheath, 1984). On the other hand, the genus *Sirodotia* should be reduced to a section of the genus *Batrachospermum* (Necchi and Entwisle, 1990). There has been considerable debate as to whether these characteristics are sufficient to recognize a separate genus or just a section of the genus *Batrachospermum*. Genus *Sirodotia* together with the genera *Nothocladus* and *Tuomeya* was retained at generic level until further data are obtained (Vis and Sheath, 1998; 1999).

5.1.3. Genus Tuomeya (Harvey, 1858: 64). Type Species: Tuomeya fluviatilis (Harvey)

Genus *Tuomeya* is recognized at the generic level, even though it forms part of a well-supported clade with two *Batrachospermum* species in the *rbc*L tree (Vis et al., 1998). The sequence divergence between *Tuomeya* and the two species of genus *Batrachospermum* is 7.9–12.6%, which is greater than ranges given for intraspecific variation for these genes in other red algae, 1.2–7.2%. Furthermore, the genus *Tuomeya* has some characteristics not present in genus *Batrachospermum* having pseudoparenchymatous growth, carpogonia with oblique to perpendicular trichogyne attached to one side of the stalk, and gametophytes developing from an undifferentiated mass of cells.

5.1.4. Genus Nothocladus (Skuja, 1934: 186). Type Species: Nothocladus nodosus (Skuja)

Recognizing *Nothocladus* as a section of the genus *Batrachospermum* (Necchi and Entwisle, 1990) would be consistent with the fact that the position of *Nothocladus* is uncertain given that it is either unassociated or placed in different groupings of the various trees generated (Sheath et al., 1996). However, they retain *Nothocladus* in the Batrachospermaceae because it has a unique combination of characteristics of the Batrachospermaceae: symmetrical carpogonial base, entirely indeterminate gonimoblast filaments, and mass of postfertilization fusion cells (Sheath et al., 1996).

5.2. FAMILY PSILOSIPHONACEAE SHEATH, MÜLLER AND VIS IN (SHEATH ET AL., 1996). TYPE GENUS: GENUS *PSILOSIPHON* (ENTWISLE)

5.2.1. Genus Psilosiphon (Entwisle, 1989): Type Species: Psilosiphon scoparium (Entwisle)

Psilosiphon scoparium is not closely allied with the taxa of the Lemaneaceae, lending support to the new proposed monogeneric family Psilosiphonaceae (Sheath et al., 1996). Based on the ultrastructural features, they concluded that *Lemanea* and *Paralemanea*, while they are distinct from each other, are closely related in their morphological construction, ultrastructure, and reproduction and should remain in the Lemaneaceae. In contrast, *Psilosiphon* is quite different from these two genera warranting a new family. Analysis of the *rbc*L and 18S rRNA

genes supports the separation of genus *Psilosiphon* from genera *Lemanea* and *Paralemanea* (Vis et al., 1998).

5.3. FAMILY LEMANEACEAE (AGARDH, 1824). TYPE GENUS: GENUS *LEMANEA* (BORY)

Family Lemaneaceae consists of two genera, *Lemanea* and *Paralemanea.* Following analysis of the *rbc*L and 18S rRNA gene sequences, the two genera, *Lemanea* and *Paralemanea*, were retained pending further investigation, but the possibility that *Paralemanea* is paraphyletic was noted (Vis et al., 1998).

5.3.1. Genus Lemanea (Bory de Saint-Vincent, 1808). Type Species: Lemanea fluviatilis (Linnaeus) (C. Agardh)

Axial cells lack cortical filaments; ray cell is T- or L-shaped, closely applied to outer fascicles; spermatangial papillae in circular patches on node.

5.3.2. Genus Paralemanea (Vis and Sheath, 1992). Type Species: Paralemanea catenata (Kützing) (Vis et Sheath)

Axial cells are with abundant cortical filaments, ray cell is simple, not abutting outer fascicles; spermatangial papillae in rings around node.

Genus *Paralemanea* is paraphyletic by the *rbc*L gene sequence, but its position is equivocal in the 18S rRNA gene analysis, because only one species was sequenced. On the basis of the *rbc*L molecular phylogeny, *Paralemanea* cannot be recognized at any taxonomic level. However, because *Paralemanea* is well defined morphologically, it is defined at generic level pending further evidence from nuclear gene sequences. Clearly, the intrageneric, familial classification of family Lemaneaceae must be revisited (Vis et al., 1998).

5.3.3. Genus Petrohua (G.W. Saunders in Vis et al., 2007). Type Species: Petrohua bernbei (G.W. Saunders in Vis, Harper and Saunders)

Petrohua is established from Chile (Vis et al., 2007). Genus *Petrohua* according to the analyses provides an interesting insight into the development of a gross morphology consisting of a pseudoparenchymatous tube. It would have been expected from morphology and internal anatomy that the genus would have been closely allied to and share a recent common ancestor with *Lemanea* and *Paralemanea*, but that was not shown in the combined analyses of the *rbc*L and the 26S rRNA gene sequences (Vis et al., 2007).

5.3.4. Genus Balliopsis (Saunders and Necchi. 2002). Type Species: Balliopsis prieurii (Saunders and Necchi)

Molecular analysis of *Ballia callitricha*, the type species of genus *Ballia*, showed only a remote relationship to order Ceramiales (Choi et al., 2000). Distance and parsimony analyses based on nuclear 18S rRNA sequences of the type species and two additional marine species of genus *Ballia* revealed that they represent a distinct

lineage sister to order the Acrochaetiales, order Batrachospermales, order Nemaliales, and order Palmariales. As *Ballia callitricha* is the type species of the genus *Ballia* and is distinct in anatomical and molecular features from all recognized orders, a new order and family (order Balliales and family Balliaceae) were proposed. The relationship of marine species and freshwater species is uncertain (Kumano and Phang, 1990; Choi et al., 2000). It is clear that the two recognized freshwater species of the genus *Ballia*, *Ballia. Pinnulata*, and *Ballia prieurii*, need to be reexamined. In fact, an analysis of nuclear small-subunit and partial large-subunit rDNA sequences from a freshwater species, *Ballia prieurii*, shows that it is related to the Batrachospermales rather than the Balliales, so that a new genus *Balliopsis* is proposed within order Batrachospermales. Owing to the obvious problems with the family Batrachospermaceae being paraphyletic, a formal familial proposal was avoided and *Balliopsis* was left as a genus *incertae sedis* in the Batrachospermales (Saunders and Necchi, 2002).

6. Order Thoreales (Müller, Sheath, Sherwood, and Pueschel in Müller et al., 2002). Type Family: Family Thoreaceae

6.1. FAMILY THOREACEAE (REICHENBACH) (HASSALL, 1845). TYPE GENUS: GENUS *THOREA* (BORY)

The phylogenetic trees generated from *rbc*L and 18S rRNA analyses for the two genera, *Thorea* and *Nemalionopsis*, showed the family Thoreaceae to be contained in a well-supported monophyletic clade separate from the three families, family Batrachospermaceae family Lemaneaceae, and family Psilosiphonaceae, currently classified in order Batrachospermales.

Pit plugs have two cap layers, the outer one of which is usually plate-like. These findings indicate these two genera should be placed in their own order, Thoreales. Thus, the order Thoreales with two recognized genera, *Thorea* and *Nemalionopsis*, was proposed (Müller et al., 2002).

7. Order Hildenbrandiales (Pueschel and Cole, 1982) Type Family: Family Hildenbrandiaceae (Rosenvinge)

7.1. FAMILY HILDENBRANDIACEAE (ROSENVINGE 1918) TYPE GENUS: GENUS *HILDENBRANDIA* (NARDO)

7.1.1. Genus Hildenbrandia (Nardo, 1834). Type Species: Hildenbrandia rubra (Sommerfelt) (Meneghini)

Based on morphometric analysis and phylogenetic analysis of sequences of the *rbc*L and 18S rRNA genes, three groups of species of genus *Hildenbrandia* were distinguished: group 1 with freshwater species, *H. angolensis* (North America, Europe) and *H. rivularis* (Europe), group 2 with parallel tetrasporangial division,

H. occidentalis, and group 3 with nonparallel tetrasporangial division, *H. rubra,* but it is clear that they are closely related ones according to the type of pit plug structure (Sherwood and Sheath, 1998, 1999, 2000; Saunders and Bailey, 1999).

On the basis of the results of various analyses such as DAPI staining, TEM analysis, and 18S r DNA analysis, it is obvious that *Pleurocapsa cuprea* Hansgirg (1892) described as a cyanobacterial species must be classified to the Rhodophyta, close to the freshwater group of *H. rivularis and H. golensis* as a new species, *H. cuprea* (Caisova and Kopecky, 2008).

8. Order Ceramiales (Oltmanns, 1904) Type Family: Family Ceramiaceae (Dumortier, 1822)

8.1. FAMILY DELESSERIACEAE (BORY DE SAINT-VINCENT, 1808). TYPE GENUS: GENUS *DELESSERIA* (LAMOUROUX)

8.1.1. Genus Caloglossa (Harvey) (Martens, 1869). Type Species: Caloglossa leprieurii (Montagne) (Martens)

Analyses of *rbc*L gene sequences have been used for phylogeny, evolutionary divergence, biogeography, and populations of genus *Caloglossa* at species level (Kamiya et al., 1998, 1999, 2000). Although most species of *Caloglossa* have been known from brackish habitat, some are reported from freshwater habitat. For example, *Caloglossa ogasawaraensis* is reported from Japan, India, and Malaysia: *C. beccarii* from Malaysia and Indonesia; *C. leprieurii* from Australia and Puerto Rico (Kumano, 2002).

8.2. FAMILY RHODOMELACEAE (ARESCHOUG, 1947). TYPE GENUS: GENUS *RHODOMELA* (C. AGARDH)

8.2.1. Genus Bostrychia (Montagne). Type Species: Bostrychia scorpioides (Hudson) (Montagne)

Analyses mainly using *rbc*L and *rbc*S genes have been made to determine relationship among genus *Bostrychia* at species level (Zuccarello and West, 1997; Zuccarello et al., 1999a, b). Most taxa of *Bostrychia* have been known from brackish habitat, but some are known from freshwater habitats. For example: *B. moritziana* is reported from Brazil; *B. simpliciuscula* from Japan and Tonga; *B. flagellifera* from Japan, New Zealand, and Australia (Kumano, 2002).

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PART 3:
GENOMIC STUDIES
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Biodata of **Elisabeth Gantt**, communicating author of ***"Porphyra: Complex Life Histories in a Harsh Environment: P. umbilicalis, an Intertidal Red Alga for Genomic Analysis"***

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PORPHYRA: COMPLEX LIFE HISTORIES IN A HARSH ENVIRONMENT: *P. UMBILICALIS, AN INTERTIDAL RED ALGA FOR GENOMIC ANALYSIS*

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1. Introduction

The red algal genus *Porphyra* ("nori," "laver") is species-rich and widely distributed (Brodie and Zuccarello, 2007). The gametophyte is characterized by a large, foliose blade or thallus, which in many locations alternates with a small, filamentous sporophyte (the shell-boring "conchocelis" phase). The reproductive phases among the *Porphyra* species can vary greatly, with essential details of reproductive life-histories summarized in Brodie and Irvine (2003) and Fig. 1. The life history of *Porphyra* was elucidated by Kathleen Drew (1949), who first demonstrated alternation between the blade and conchocelis phases in a British isolate of *Porphyra*, which she initially identified as *P. umbilicalis* but which was almost certainly *P. dioica* (Brodie and Irvine, 2003; Brodie et al., 2008). Drew's incisive observations allowed for greatly improved production of the economically important *P. yezoensis* (nori) in Japan, beginning in the 1950s (Ueda, 1958; as cited in Kafuku and Ikenoue, 1983).

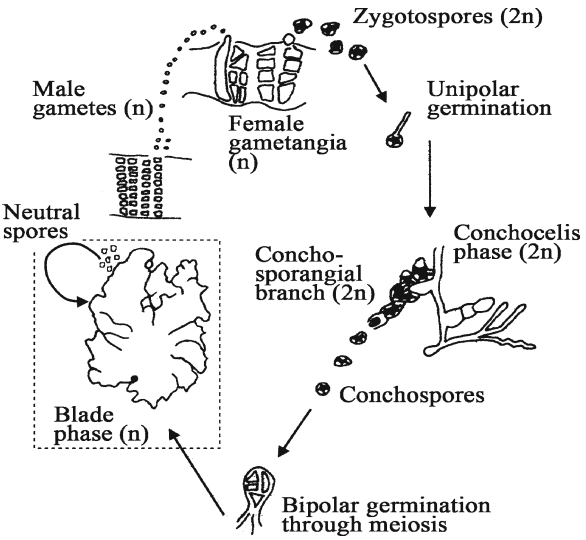

Figure 1. Heteromorphic, sexual life history of *Porphyra*. Both phases of the life history produce spores that regenerate each phase directly. The boxed stage is a focus of the *Porphyra* genomics' project (courtesy of M. Holmes and J. Brodie).

Table 1. Classes of Rhodophyta showing evolution of key characteristics in Bangiophyceae.

Class sensu Yoon et al. (2006)	Unicellular (U) or multicellular (M)	Asexual spores	Sexual reproduction	Complex life histories	Pit plugs
Cyanidiophyceae	U	–	–	–	–
Porphyridiophyceae	U	–	–	–	–
Rhodellophyceae	U	–	–	–	–
Compsopogonophyceae	M	Present	Present	–	–
Stylonematophyceae	M	Present	Present	–	–
Bangiophyceae	M	Present	Present	Present	Present
Florideophyceae	M	Present	Present	Present	Present

Today, the aquaculture crop of nori in Japan, China, and Korea is worth more than $1.4 billion/year (Yarish and Pereira, 2008). *Porphyra* is valued as human food because of its high level of protein (25–50%), vitamins (B_{12}, C), trace minerals, and dietary fiber (Noda, 1993; MacArtain et al., 2007). *Porphyra umbilicalis* is currently a target of pilot aquaculture in North America (Blouin et al., 2007).

Porphyra belongs to the order Bangiales (Bangiophyceae) (Table 1), which represents an ancient lineage with fossil records that provide evidence for sexual reproduction that dates to at least 1.2 BYA (Butterfield, 2000). Traditionally, *Porphyra* species have been largely delineated on the basis of blade characteristics (e.g., pigmentation, blade thickness, cell dimensions), but recent molecular analyses suggest that the genus is polyphyletic (Oliveira et al., 1995; Nelson et al., 2006). The filamentous (*Bangia*) and blade-like forms (*Porphyra*) of the Bangiophyceae exhibit convergent functional and morphological tendencies, which appear to have arisen numerous times during the evolution of these genera.

It is generally accepted that multicellularity arose independently in different major eukaryotic lineages (Baldauf, 2008). Detailed investigations indicate that many genetic building blocks critical for attaining multicellularity are shared by animal and plant model organisms. Some key innovations thought to have been important in the radiation of developmental patterns in red algae were already present in the common ancestor of *Porphyra*, while others had yet to develop. For example, pit plugs (= pit connections) are a strongly conserved feature of red algae, and are thought to have played important roles in the evolution of structural diversity, reproductive strategies, and ecological adaptation. Pit plugs are always present in the florideophyte red algae, where variations in their ultrastructure are highly informative in ordinal-level systematics (Pueschel, 1994). The relative importance of pit plugs to red algal metabolic circuits, core physiological functions, and evolutionary processes are largely unknown. Although pit plugs are invariably present in florideophytes, simpler and presumably ancestral forms of this structure are found in the conchocelis phase of members of the Bangiales (Bourne et al., 1970; Lee and Fultz, 1970; Ueki et al., 2008). Thus, comparative transcriptomics of gametophyte and sporophyte stages of *Porphyra* offer an unparalleled opportunity to identify genes encoding pit plug structural elements and to unravel genetic and metabolic networks involved in their biogenesis.

2. An Emphasis on the Genome of *Porphyra umbilicalis*

To date, the only red algal genomes for which there are complete or nearly complete sequences are the acidothermophiles *Cyanidioschyzon merolae* (Matsuzaki et al., 2004; Nozaki et al., 2007) and *Galdieria sulphuraria* (Barbier et al., 2005). These algae belong to the Cyanidiophyceae, which has most likely experienced extensive genome reduction and elevated gene divergence rates as a result of adaptation to the specialized, extreme habitats of the fumaroles and acidic waters in which they live. The genome of *C. merolae* (16.5 Mb) is highly compact with a low number of protein-encoding genes (~5,300), relatively few transposable elements (Nozaki et al., 2007), and the occurrence of introns in only 26 genes (Matsuzaki et al., 2004). The haploid genome of *P. purpurea* was determined by flow cytometry to be ~270 Mb (using nuclei isolated from protoplasts, Le Gall et al., 1993), a value that is many times larger than the genome sizes of *C. merolae* and *G. sulphuraria*. Therefore, it is reasonable to assume that the *Porphyra* genome contains many more protein-encoding genes than are present in these highly modified, unicellular taxa. The genomic complexity of *Porphyra* is expected to be more like that of free-living, mesophilic rhodophytes.

The chromosome number of *Porphyra* species tends to be low when compared with that of many other red algae. The haploid (n) chromosome number of *P. umbilicalis* is reported to range from 3 to 5 (rf. Table 4.1 in Cole, 1990), which may be correlated with the geographic distribution of different isolates, cryptic taxa, or misidentifications. Brodie and Irvine (2003) found n=4 in *P. umbilicalis* from British shores, and *P. purpurea* (the "type species") also has four chromosomes, whereas *P. yezoensis* generally has three (Wilkes et al., 1999). We chose to generate genomic DNA from a strain of *P. umbilicalis* that is abundant on the northeastern coast of the USA, because the blade reproduces asexually by neutral spores, making it possible to obtain large quantities of homogeneous genetic material. The imminent sequencing of the *P. umbilicalis* genome by the Joint Genome Institute and the subsequent analyses of its sequence will provide us with insights into (1) mechanisms associated with the transition from a unicellular life mode to one of multicellularity, including regulatory elements critical for that transition, (2) the development of the sexual phases of the life cycle, and (3) adaptive mechanisms that enable the organism to cope with physiological stresses, including desiccation, high light, and nutrient deprivation.

3. Asexual Reproduction

Four types of asexual spores are produced by the *Porphyra* blade (Nelson et al., 1999); they include archeospores, neutral spores, agamospores, and endospores. Understanding and exploiting asexual reproduction can be highly beneficial for commercial cultivation of *Porphyra*. For example, the production of asexual

spores results in an increased "set" of gametophytic thalli on the cultivation nets and allows for a longer cultivation period. Also, archeospores can be used for establishing diverse, valuable lines, including mutants that have a desirable texture or color. The different spore types are described below:

1. An archeospore (= monospore in older literature) is formed by differentiation of a vegetative blade cell into a single spore, which germinates into the blade/foliose phase. Some species of *Porphyra* form archeospores only on young thalli a few millimeters long, whereas other *Porphyra* species (e.g., *P. yezoensis*) continue producing archeospores on thalli several centimeters long. Recently, Kitade et al. (2008) reported the identification of candidate genes involved in asexual reproduction in *P. yezoensis*.
2. Neutral spores are formed by mitotic cleavage of blade cells; such spores germinate and develop into new blades. Gametophytic *P. umbilicalis* regenerates the blade directly through the production of neutral spores throughout the year on the coast of Maine. The viability of these spores declines in summer, and sexual reproduction has not been observed in this population (Blouin et al., 2007), in contrast to northeastern Atlantic *P. umbilicalis* where both sexual and asexual reproduction are observed (Brodie and Irvine, 2003).
3. Agamospores are formed by mitotic cleavage of blade cells, without fertilization, and under appropriate conditions may germinate into conchocelis filaments.
4. Endospores are formed by mitotic divisions of blade cells, and occur as an irregularly arranged and indefinite number of spores encased in a distinct envelope.
5. Two types of asexual spores (neutral conchospores and archeospores) that develop from the conchocelis stage are also known (Knight and Nelson, 1999; Nelson et al., 1999).

4. Genome Evolution

Genome evolution is a complex process, characterized by a series of sometimes overlapping evolutionary events. Lateral gene transfer (LGT, the transfer of genes between two different strains or species) is a ubiquitous phenomenon whereby exogenous genetic material is taken into an organism and is subsequently stably incorporated into its genome. The contribution of LGT to genome innovation and physiological diversity of life forms has been highlighted in recent years (Boucher et al., 2003). Using a phylogenetic approach for multigenomic analysis, previous studies have demonstrated that LGT is prominent among prokaryotes and some eukaryotes (Keeling and Palmer, 2008). Because many algae have lost their ancestral phagotrophic capacity, the extent of LGT between algae and other species or lineages may be limited, but LGT in algae has been noted in a number of studies, most of which involve intron sequences (e.g., between brown and red algae [Bhattacharya et al., 2001]; between fungi and red algae [Müller et al., 2005]). A prime example of LGT is provided by the location of genes that regulate plastid structure and function,

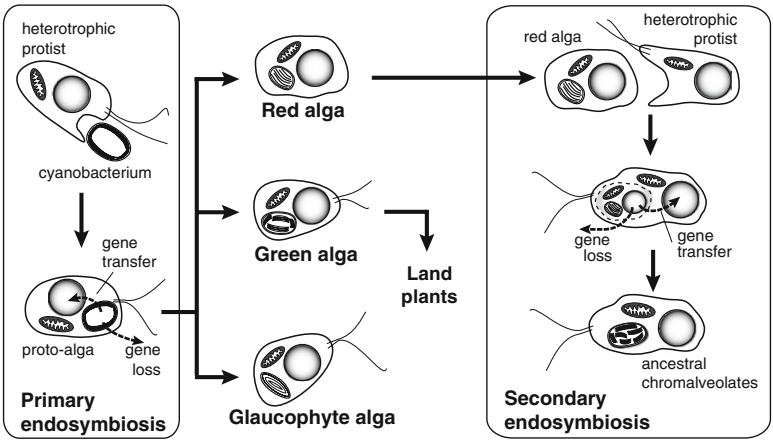

Figure 2. Endosymbiosis and gene transfer model leading from cyanobacteria to the red algae through primary endosymbiosis (*left*), and subsequently, through secondary endosymbiosis to other algal lineages including brown algae, cryptomonads, diatoms, dinoflagellates, haptophytes, and apicomplexans. Each transfer also resulted in gene losses (courtesy of Cheong Xin Chan).

which evolved from the ancestral genes of the engulfed primary endosymbiont, a cyanobacterium; these genes are now present in both the red algal chloroplast and nucleus (Fig. 2). Subsequent secondary and even tertiary endosymbiotic events produced additional algal lineages and led to further gene transfers in the new host organism (Reyes-Prieto et al., 2007).

The much larger size and, presumably, greater number of genes in the genome of *P. umbilicalis* compared to *C. merolae* will provide additional information to test the idea of Plantae monophyly. This includes a better knowledge of:

1. The diversity of genes encoding enzymes of common metabolic pathways that function in plastids of *Porphyra* and other Plantae members (e.g., amino acid, lipid, and cofactor biosynthesis)
2. LGT between *Porphyra* and other algal lineages (e.g., Calvin-Benson Cycle enzymes in plastids; Reyes-Prieto and Bhattacharya, 2007)
3. Putative gene fusions that may have occurred in the chromosome of the ancestral host prior to the radiation of the Plantae (Gross et al., 2008)

Other phylogenetically relevant genes are those that encode products that function in plastids (Fig. 2) and that are common to plants and algae of the green, red, and glaucophyte lineages, but absent in cyanobacteria. These genes originated *de novo* from the chromosome of the host or were recruited from pre-existing genes to express a new function in the organelle. Examples that can be examined in the *P. umbilicalis* genome include the light-harvesting protein family, solute transporters on the inner plastid membrane, and the TOC/TIC plastid protein translocons (Kalanon and McFadden, 2008).

Table 2. Genome size (Mb) and percentage of the genome corresponding to transposable elements (TE).

Organism	Assembly size (Mb)	TE (~genome %)
Ostreococcus tauri	12.6	10
Cyanidioschyzon merolae	16.5	0.7
Thalassiosira pseudonana	34.5	2
Arabidopsis thaliana	140.1	15
Oriza sativa	430	14
Homo sapiens	3,000	44

Transposable elements (TEs) are believed to play a fundamental role in genome evolution, adaptation to diverse environmental conditions and speciation (Kazazian, 2004), and to be a major source of variation in genome size among eukaryotes. TEs are considered mobile elements, able to move to other locations within the genome. At this juncture, there is limited evidence of a linear relationship between the total number of transposable elements in the genome of an organism and genome size (Kidwell, 2002); however, it is still likely that the ~270 Mb genome of *P. umbilicalis* will have more TEs than the relatively few that are found in *C. merolae* (Table 2.). The number and features of TEs in the *P. umbilicalis* genome will augment our limited understanding of the role of these elements in red algal evolution.

It will be important to determine the genome content and possible regulatory roles of small RNAs (~20–40 nt RNAs) in *P. umbilicalis*. Small RNAs can regulate gene expression through a variety of mechanisms, including RNA decay, DNA methylation, or translation efficiency (Wu and Belasco, 2008; Vazquez, 2006). Small RNAs (for example microRNAs [miRNA], and small-interfering RNAs [siRNA]) are incorporated into protein complexes (e.g., Argonaute/Piwi proteins, with PIWI and PAZ domains) that ultimately control their regulatory function (Takeda et al., 2008). When a BLAST search was conducted (L. Rymarquis, unpublished) against the ~30,000 sequences from various *Porphyra* species present in the NCBI database, it was observed that several ESTs of *P. yezoensis* could potentially encode proteins containing the PIWI and PAZ domains characteristic of Argonaute/Piwi family proteins. Analyses of such sequences, as well as small RNA sequences, will ultimately determine what types of small RNAs, if any, are produced by *P. umbilicalis* and may provide clues to their mode of action.

5. Associations with Other Organisms

Various types of associations between red algae and other organisms have been noted, but are still little studied. Some associations are disadvantageous, such as the pathogenic responses elicited when cultivated *Porphyra* is infected by the oomycete *Pythium porphyrae*. This fungus, which is the causative agent of red rot

disease in *Porphyra*, can infect and encyst on many different red algae, as shown by infection experiments of Uppalapati and Fujita (2000). Zoospores attach to *Porphyra* and *Bangia*, with a successful invasion of the algal tissue being dependent on sulfated galactans (porphyran, commercial agar, agarose, and carrageenans).

Vitamin B_{12} is needed as a growth factor by several algal species, serving as a cofactor for methionine synthase (METH). Croft et al. (2005) reported that the vitamin could be supplied by symbiotic bacteria. On the other hand, Droop (2007) has proposed that in the natural environment, there is sufficient cobalamin in seawater for algal growth. Whether or not *Porphyra* requires exogenous B_{12} vitamins for growth remains to be determined, but Takenaka et al. (2003) found that axenically grown *P. yezoensis* contained nearly the same concentration of B_{12} as wild plants. Some red algae do not require B_{12}; for example, *Porphyridium purpureum* (*P. cruentum*) has been grown axenically in a chemically defined artificial seawater medium for decades (E. Gantt, personal observation). Genomic analysis of *Cyanidioschyzon merolae* (Matsuzaki et al., 2004) revealed that it encodes an alternative, B_{12}-independent form of methionine synthase, METE. The *Porphyra* sequencing project will provide the first genomic information about the role of B_{12} in a multicellular red alga, and may have implications in farming of seaweed for food, and/or for the production of vitamins.

6. Cytoskeletons, Cell Division, and Flagellar Genes

Forty-four different cell-cycle genes and ten different cytoskeletal genes have been identified from the existing *P. yezoensis* EST data set (S. Lin and Y.Y. Zhuang, unpublished analysis). The cell-cycle-related genes include core regulatory genes such as cyclin kinases (*cdc2* and other CDKs), CDK regulators, cyclins (e.g., cyclin E, which regulates the transition from G_1 to S phase; cyclin B, which regulates the G_2 to M transition), and the anaphase promotion complex proteins, plus a proliferating cell nuclear antigen (PCNA). Phylogenetic analysis reveals a high divergence of these genes across different algal groups and other organisms. According to this analysis, *Porphyra* does not appear to be particularly ancient with respect to the core components of the cell cycle, although information on genetic regulatory networks that control cell cycle progression and cytoskeletal function in algae is still very limited, particularly for *Porphyra*.

Red algae have no flagella or basal bodies (centrioles), structures involved in motility, mitosis, and sensory functions in many organisms. These structures are considered to be features of the ancestral protist that gave rise to the red algal lineage, but have been lost during red algal evolution, as also occurred in some green algae and during the evolution of land plants (Merchant et al., 2007). Based on this hypothesis, red algal genomes may contain remnant genes or pseudogenes that encode sequences that resemble flagellar and basal body proteins. Tubulin and kinesin are known from *Cyanidioschyzon*, but dynein is absent, consistent with the absence of a flagellum.

Many red algal spores (e.g., archeospores, tetraspores, carpospores; see Pickett-Heaps et al., 2001) exhibit an amoeboid type of cell motility, which has also been observed in neutral spores of *P. umbilicalis* (Brodie and Irvine, 2003; N. Blouin, personal observation). Analysis of this motility in archeospores of *Porphyra pulchella* (Ackland et al., 2007) demonstrated that pseudopodial activity was dependent on an actin/myosin cytoskeleton, with the actin filaments arrayed in short and long peripheral bundles.

7. Photosynthesis, and Light Absorbing Molecules

The only chlorophyll present in *P. umbilicalis*, as in other red algae, is chlorophyll *a*. Earlier reports claiming the presence of chlorophyll *d* in certain red algae are now thought to reflect the adherence of chlorophyll *d* producing cyanobacterial epiphytes to the algal surface. Enzymes required for the biosynthesis of chlorophyll *a* as ones use in green plants (Masuda, 2008) are known. Genes encoding many of these essential biosynthetic enzymes also occur in *Cyanidioschyzon* and in the cyanobacterium *Synechocystis* PCC 6803. While very likely, it remains to be determined whether *P. umbilicalis* employs a similar biosynthetic pathway to chlorophyll *a*.

It is very likely that the plastid genome of *P. umbilicalis* is very similar to that of its relative *P. purpurea,* which has a chloroplast genome of about 191 kb (Reith and Munholland, 1995), larger than that of *Cyanidioschyzon* (ca. 150 kb) (Ohta et al., 2003). Current estimates predict that the plastid genome of *Porphyra* encodes over 200 proteins one third of which are not present in the *Arabidopsis* chloroplast genome (Reith and Munholland, 1995). Despite the large size of the red algal chloroplast genome and its cyanobacterial origin, several notable genes expected to reside on the plastid genome have not been found; these include *ndh* (energy conversion protein), *infA* (translation factor), and *clpP* (ATP-dependent protease). The genes encoding reaction center proteins of photosystem (PS) I and II, and most of the genes encoding phycobilisome polypeptides (major light harvesting complex of PS II, see later) are encoded on the chloroplast genome.

In *P. umbilicalis*, the major PS II antenna, the phycobilisomes, are generally ellipsoidal in shape (Algarra et al., 1990). The predominant pigment within phycobilisomes is rhodophycean phycoerythrin (R-PE) with absorbance maxima (A_{max}) at ca. 498, 542, and 565 nm. R-phycocyanin (A_{max} of 553 and 615 nm) and allophycocyanin (A_{max} of 650 nm) are important but occur in relatively lower amounts, which is rather typical for PE-containing rhodophytes (Gantt et al., 2003). Functionally significant for extended light absorption is the development of chlorophyll-carotenoid binding proteins (LHC I) associated with PS I (Wolfe et al., 1994). Because small proteins with domains having homology to LHC I polypeptides are present in cyanobacteria (Dolganov et al., 1995), it is assumed that following the primary endosymbiosis, fusion of genes encoding these cyanobacterial proteins (as well as the movement of the genes to the nuclear genome of the host) was critical for the evolution of the LHC gene family. Phycobilisomes in *Porphyridium purpureum (cruentum)*

are primarily, if not exclusively, associated with PS II, and only secondarily transfer excitation energy to PS I. Enhancing the light absorbance capacity for both photosystems represents a significant evolutionary advantage. The association of LHCs directly with the PS I reaction centers was established in *Porphyridium* (Wolfe et al., 1994), but can be expected to occur in other red algae based on comparisons of LHC protein sequences and the occurrence and amount of pigments that function in light absorption (Gantt et al., 2003).

Red algae are relatively diverse in the composition of their carotenoids, pigments that may both enhance light absorbance capacity and protect against photo-oxidation. The red alga *C. merolae* synthesizes only a few β-ring carotenoids (i.e., β-carotene and zeaxanthin; Cunningham et al., 2006). Other red algae produce carotenoids with ε-rings as well as β-rings (i.e., α-carotene [β, ε-carotene] and lutein), and still others accumulate epoxycarotenoids such as antheraxanthin and violaxanthin (Marquardt and Hanelt, 2004; Schubert et al., 2006). Lutein and ß-carotene are the predominant carotenoids of *P. umbilicalis* (Fig. 3). and of other species of *Porphyra* (Shimma and Taguchi, 1966; Schubert et al., 2006), with lesser amounts of α-carotene and zeaxanthin also typically observed. Epoxycarotenoids such as antheraxanthin and violaxanthin were not detected in a recent analysis (Fig. 3), and have not been identified in other *Porphyra* species (Schubert et al., 2006). Interestingly, the lutein in dried *P. yezoensis* was reported to be present largely as a cis-geometrical isomer (Shimma and Taguchi, 1966), which is potentially an artifact associated with drying of the samples.

Of particular interest regarding the carotenoid pathway of *P. umbilicalis* is the identity of the enzyme catalyzing the formation of ε-rings. The ε-ring cyclase of plants and green algae is thought to have originated by duplication of a gene encoding a ß-ring cyclase, and plant ε-cyclase enzymes retain some ability to

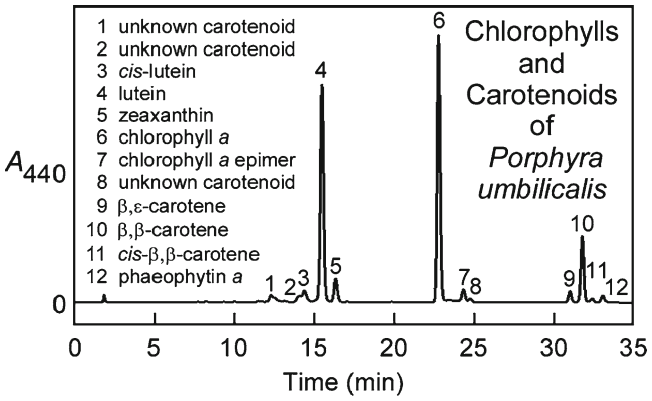

Figure 3. HPLC elution profile of lipid-soluble pigments extracted from a *P. umbilicalis* thallus from the coast of Maine. Extraction and analysis was as in Cunningham et al. (2006), except the mobile phase gradient was 50% B (ethyl acetate) in A (acetonitrile/methano/triethylamine, 90/10/0.1) (courtesy of F.X. Cunningham).

produce β-rings as well as ε-rings (Cunningham et al., 2006). Also of interest is the origin of the enzyme or enzymes responsible for 3-hydroxylation of carotenoid β- and ε-rings, thereby enabling the synthesis of zeaxanthin and lutein. *Cyanidioschyzon merolae* has a plastid gene encoding a polypeptide similar in sequence to cyanobacterial CrtR-type ß-carotene 3-hydroxylases, but the chloroplast genomes of *P. yezoensis* and *P. purpurea* do not have such a gene, and a gene encoding a polypeptide similar to CrtR or to plant or green algal CrtZ-type ß-carotene 3-hydroxylases is not apparent in the available *Porphyra* EST sequences.

Exposure to high light, especially when coupled with desiccation or nutrient limitation, can cause severe damage to the photosynthetic apparatus as well as to other cellular constituents and processes. This damage results largely from the accumulation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Halliwell and Gutteridge, 1999). The seasonal production of some antioxidants by *P. umbilicalis* was studied by Sampath-Wiley et al. (2008). These workers observed an accumulation of the highest levels of antioxidants during the summer, with levels of glutathione reductase, catalase, and carotenoids being higher during times of emersion relative to immersion. Diffusion could also play a role in eliminating H_2O_2 from *Porphyra* because of its thin blade (Collén et al., 1995). Analysis of the genome of *P. umbilicalis* will help researchers decipher potential mechanisms by which this alga, and potentially other desiccation-tolerant, high-light-resistant algae can cope with extremely high excitation pressure.

Mycosporine-like amino acids (MAAs) provide protection against UV radiation that can damage the cell's DNA and photosynthetic apparatus (Shick and Dunlap, 2002; Sinha et al., 2007). While *Porphyra* spp. can synthesize a number of different MAAs (shinorine and porphyra-334; Takano et al., 1979; Sinha et al., 2007), the details of their synthesis and their precise intracellular functions remain to be determined. More work is required to understand how the rich arrays of light-absorbing molecules in the red algae are co-regulated.

8. Carbohydrates: Storage and Cell Wall Composition

Unlike other photosynthetic eukaryotes that synthesize and store carbohydrate reserves in the plastid, red algae synthesize and store starch in the cytoplasm (Viola et al., 2001). This "floridean" starch consists of a backbone of α-1,4 linked glucan with α-1,6 branches, and it is structurally more similar to amylopectin than to glycogen. Floridoside (2-O-α-D-galactopyranosylglycerol) is the main source of soluble storage carbon in red algae, with a carbon transport function much like that of sucrose (Viola et al., 2001). Like sucrose, floridoside is synthesized via a phosphorylated intermediate in the cytosol. The genes for enzymes in floridoside metabolism have been identified in *C. merolae* and *G. sulphuraria* (Barbier et al., 2005), but are yet to be characterized in *Porphyra.* In *Gracilaria tenuistipitata*, the nucleotide sugar UDP Glucose (UDPGlc) appears to be the preferred substrate

for synthesis of floridean starch. The synthesis and accumulation of floridean starch present something of an evolutionary puzzle: features of its synthesis (from UDPGlc in the cytoplasm) are similar to features of glycogen synthesis in fungi and animals, whereas the structure of floridean starch is more akin to the carbon reserves accumulated in the plastids of chlorophytes.

The inner cell walls of the blade phase of the *Porphyra* thallus are reticulate and multilayered with xylan microfibrils in an amorphous matrix; these provide structural support for the thallus. The outer wall matrix is less structured and consists largely of porphyran (up to 30% dry wt.), a linear polymer of galactans that are sulfated to different degrees (Mukai et al., 1981). On the outer surface of the cell wall, a morphologically distinct cuticle of neutral polysaccharides is found; the cuticle is also protein-rich, unlike the waxy cuticle of vascular plants. The polysaccharide chains associated with the cell wall are synthesized in the Golgi complex from sugar nucleotide units (Cole et al., 1985).

An interesting evolutionary question is the occurrence of crystalline cellulose in the filamentous conchocelis phase of *P. leucosticta* (Gretz et al., 1986), and its absence in the gametophytic blade. In red algae in general, and in *Porphyra* in particular, different polymers fulfill the same function in the cell wall in different cell types or at different phases of the life cycle. Understanding the biosynthesis of the *Porphyra* cell wall over its life cycle and the specific functional associations of the individual wall components may provide important insights into the dynamics of cell wall function, structure, and biosynthesis, especially as they relate to the daily hydration extremes experienced in the intertidal zone. Genomic studies will also contribute to our understanding of the evolution of cellulose synthesizing enzymes.

9. Circadian and Circannual Rhythms

Porphyra species occupy distinct areas of the intertidal and subtidal zones, which suggests that different species may have different tolerances for water loss. *P. umbilicalis* is common in the high and mid-intertidal zones, and as is true of many intertidal *Porphyra* species, it dries to a fraction of its wet weight during daytime low tides. The molecular mechanisms for desiccation tolerance are currently unknown, but some may be regulated by an internal clock. Anticipating predictable environmental changes, on both diurnal and seasonal time scales, would prepare *Porphyra* for rhythmic changes in the environment. Indeed, growth and cell division occur principally in the dark phase of a 16:8 L:D cycle, and *P. umbilicalis* cells enter prophase near the end of the light period (Lüning et al., 1997). These rhythms persisted in constant light, and the free-running rhythm found for growth under constant green or red light was observed to be intensity-dependent, whereas growth under constant blue light was arhythmic (Lüning et al., 1997; Lüning, 2001). Lüning (2001) suggested that the circadian oscillator was especially sensitive to blue light but uses all visible wavelengths in synchronization of the rhythm. The existing data supported cryptochrome, phytochrome, and/or

opsins as potential photoreceptors for the rhythm (Lüning, 2001). *Porphyra* also undergoes many developmental transitions (e.g., gametophytic to sporophytic transitions in the life cycle) that are regulated by photoperiod (e.g., Tseng, 1981). Rapid progress in identification of the photoreceptors involved in these processes should be possible when the draft genome of *P. umbilicalis* becomes available.

10. Nutrient Acquisition

The *Porphyra* blade is one or two cells thick (a species-dependent character), which provides the organism with an extremely high surface area to volume ratio for rapid nutrient uptake (Kraemer et al., 2004). Interestingly, Kim et al. (2007) found that *P. umbilicalis* was able to take up nitrate from the surrounding medium more rapidly than several species with thinner thalli. Nitrogen uptake experiments on *P. yezoensis* and *P. purpurea* found that NH_4^+ was preferred over other nitrogen sources, such as NO_3^- (Kraemer et al., 2004). This information is particularly relevant for mixed aquaculture designs. An understanding of mechanisms involved in nitrogen acquisition in *Porphyra* is clearly important from an economic perspective, because *Porphyra umbilicalis,* and several other species, have been used in demonstration-scale, integrated multitrophic aquaculture systems (IMTAs) in the northeastern US. *Porphyra* functions as an extractive component of aquaculture systems by removing ammonium and other finfish wastes that serve as required nutrients for the alga (Carmona et al., 2006; Blouin et al., 2007).

Many of the components involved in sulfate uptake and assimilation in *P. yezoensis* were identified from nucleotide sequences present in the EST database (www.kazusa.or.jp/en/plant/porphyra/EST/) (Asamizu et al., 2003). In *P. purpurea* and *P. yezoensis*, cDNAs were isolated that are likely to encode known sulfate transporters and enzymes involved in reductive assimilation of sulfate (adenosine 5′-phosphosulfate kinase, sulfate adenyltransferase, cysteine synthase, sulfite reductase) (Minocha et al., 2008). Furthermore, analyses of cDNA libraries offer the potential for identification of enzymes that specifically catalyze the sulfonation of carrageenans or agars, which will greatly aid in our overall understanding of the flux of sulfur metabolites in *Porphyra,* the mechanisms involved in sulfonation, and functions of sulfonated polysaccharides.

11. Future Approaches and Challenges

There are many uncertainties about the ways in which *Porphyra* cells adjust their physiology/metabolism to accommodate stresses such as desiccation or to undergo cellular differentiation and development. The imminent sequencing of the *P. umbilicalis* genome by the Joint Genome Institutes, the availability of a diversity of *Porphyra* species with different tolerances to desiccation, and the finding that tolerant species can rapidly rehydrate and activate cellular metabolism make

Porphyra the genus of choice for developing a model system to define the critical mechanisms that enable organisms to cope with extreme water loss. At this juncture, there are also many molecular and genomic tools that would provide new insights into the desiccation process and its consequences. These tools include EST sequences, which have already been generated to some extent for *P. yezoensis* and *P. haitanensis* (Nikaido et al., 2000; Lee et al., 2000; Fan et al., 2007); the construction of BAC and plasmid libraries; further generation of mutants (Miura, 1990; Niwa et al., 1993; Mitman and van der Merr, 1994; Zhang et al., 2005) and especially selected mutant libraries; the development of a transformation system (Fukuda et al., 2008); and the application of high throughput technologies for generating in-depth transcriptome information (e.g., by 454 sequencing of the transcriptome when there is no complete genome sequence and Illumina sequencing when there is a fully sequenced genome), which can be used for identifying differentially expressed genes (Pearson et al., 2001). For these reasons, and many others, the *Porphyra* genome offers unique opportunities to investigate what was probably one of the first eukaryotic forays into structural complexity. It is quite likely to provide exciting and novel insights into the evolution of adaptation processes, the control of complex developmental programs, and the establishment of multicellularity in eukaryotes.

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UTILIZING RED ALGAE TO UNDERSTAND A NEURODEGENERATIVE DISEASE

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> Among the chosen combinations, the most fertile will often be those formed of elements drawn from the domains that are far apart.
>
> Jules Henri Poincaré

1. Introduction

Lafora disease (LD; OMIM 254780), first described by the Spanish neuropathologist Dr. Gonzalo Lafora, is an autosomal recessive neurodegenerative disorder that results in severe epilepsy and death (Lafora, 1911; Lafora and Gluck, 1911). It is one of the five major progressive myoclonus epilepsies (PMEs) characterized by myoclonic, tonic seizures and progressive neurologic deterioration (Berkovic et al., 1986, 1991; Harriman et al., 1955; Hodskins, 1930). However, unlike most other forms of epilepsy, LD can only be moderately managed by medication for a brief period of time. LD commonly presents as a single seizure in the second decade of the patient's life; this single event is followed by progressive central nervous system degeneration, beginning with myoclonic seizures, followed by tonic-clonic seizures, focal occipital seizures, intellectual decline, and finally, severe motor and coordination deterioration (Acharya et al., 1995; Berkovic et al., 1993, 1991; Janeway et al., 1967; Minassian, 2001; Schwarz and Yanoff, 1965; Van Heycop Ten Ham, 1974). LD patients die within 10 years of the first seizure, usually owing to complications related to nervous-system degeneration (e.g., status epilepticus and aspiration pneumonia) (Van Heycop Ten Ham, 1974). The diagnostic hallmark of LD is the accumulation of insoluble polyglucosans called Lafora bodies (LBs) in the cytoplasm of cells in most organs (Carpenter and Karpati, 1981; Harriman et al., 1955; Schwarz and Yanoff, 1965). The size and numbers of LBs coincide with increased neuronal cell death and the number of seizures in LD patients. Thus, it is hypothesized that LBs are responsible for neuronal cell death, epilepsy, and ultimately, the death of the patient (Yokoi et al., 1968).

Recessive mutations in *EPM2B* (*epilepsy, progressive myoclonus 2b*)/*NHLRC1* account for 30–40% of LD cases (Chan et al., 2003; Ianzano et al., 2005). *EPM2B* encodes the protein malin that contains an amino-terminal RING domain and carboxy-terminal NHL domains (Fig. 1a) (Chan et al., 2003). NHL domains typically form protein-interaction motifs and RING domains are indicative of one class of E3 ubiquitin ligases (Edwards et al., 2003; Hershko and Ciechanover, 1998; Pickart, 2001; Slack and Ruvkun, 1998). Accordingly, we demonstrated that malin is an E3 ubiquitin ligase and that the NHL domains are used as a substrate interaction domain (Gentry et al., 2005). Mutations in either the RING or NHL domains results in LD (Fig. 1a).

Among the cases that are not attributed to mutations in *EPM2B*, 40–50% result from recessive mutations in *EPM2A* (*epilepsy, progressive myoclonus 2A*) (Minassian et al., 1998; Serratosa et al., 1999). *EPM2A* encodes a bimodular protein named laforin, which contains a carbohydrate binding module family 20 (CBM20) domain (Wang et al., 2002) followed by the canonical dual specificity phosphatase (DSP) active site motif HCXXGXXRS/T (Cx_5R) (Fig. 1b) (Minassian et al., 1998; Serratosa et al., 1999), and mutations in either domain lead to LD. The CBM of laforin binds to carbohydrates *in vitro* and *in vivo* (Wang et al., 2002), and

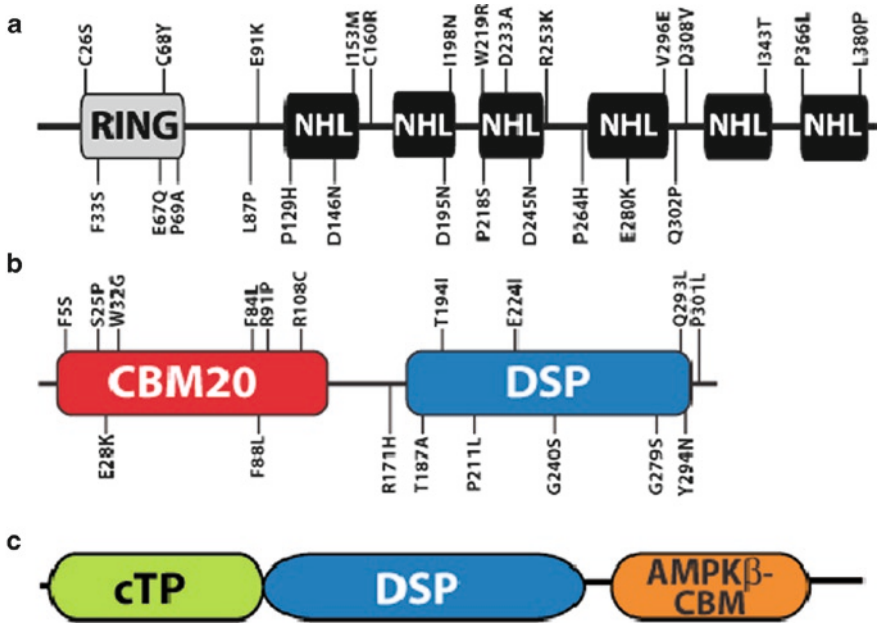

Figure 1. Schematic of (**a**) malin, (**b**) laforin, and (**c**) SEX4 proteins. The schematic of malin and laforin include point mutations from LD patients (Ianzano et al., 2005). CBM, carbohydrate binding module; DSP, dual specificity phosphatase domain; and cTP, chloroplast targeting peptide. CBM20 and AMPKβ-CBM are two different classes of CBMs.

the DSP motif can hydrolyze phosphotyrosine and phosphoserine/threonine substrates *in vitro* (Ganesh et al., 2000; Wang et al., 2002). Laforin was previously shown to be conserved only in vertebrates.

The human genome encodes 128 phosphatases (Alonso et al., 2004; Zolnierowicz, 2000), and laforin is the only one that possesses a CBM. The CBM domains are largely found in glucosylhydrolases and glucotransferases of bacterial, fungal, or plant origin (Boraston et al., 2004; Coutinho and Henrissat, 1999; Rodriguez-Sanoja et al., 2005). The vast majority of the enzymes containing a CBM binds to a specific type of carbohydrate, and utilizes a second domain to modify the carbohydrate. Accordingly, we recently demonstrated that laforin releases phosphate from the complex carbohydrate, amylopectin, while other phosphatases lack this activity (Worby et al., 2006).

2. Polyglucosans, Glycogen, Starch, and Floridean Starch

Glycogen is produced in the cytoplasm of the majority of archaebacterial, bacterial, fungal, and animal species; whereas, starch, the functional equivalent of glycogen for photosynthetic eukaryotes, is produced in a plastid (Ball and Morell, 2003). Most nonphotosynthetic eukaryotes produce glycogen from UDPglucose, while most bacteria and photosynthetic organisms synthesize glycogen or starch, respectively, from ADPglucose. Glycogen is composed of α-1,4-glycosidic linkages between glucose residues, with branches occurring in a continuous pattern every 12–14 residues via α-1,6-glycosidic linkages, and is essentially a homogeneous water-soluble polymer (Table 1). In contrast, starch is an insoluble, semi-crystalline heterogeneous mixture of 10–25% amylose and 75–85% amylopectin produced and stored within a plastid. Amylose is a linear

Table 1. Biochemical characteristics of glycogen, starch, floridean starch, and Lafora bodies.

	Organism	Building block	Residues/ branch	Branching pattern	Water soluble	Site of production
Glycogen	Eukaryotes	UDPglucose	12–14	Continuous	Yes	Cytoplasm
Glycogen	Bacteria	ADPglucose	10–15	Continuous	Yes	Cytoplasm
Starch	Plants/green algae	ADPglucose		Discontinuous	No	Plastid
–5 to 15% amylose			No branches	No branches	No	Plastid
–85 to 95% amylopectin			12–20	Discontinuous	No	Plastid
Floridean starch	Protists	UDPglucose	12–20	Discontinuous	No	Cytoplasm
Lafora body	Vertebrates	UDPglucose	12 to 30+	Discontinuous	No	Cytoplasm

molecule with very few α-1,6-glycosidic linkages. Amylopectin, like glycogen, is composed of α-1,4-glycosidic linkages with α-1,6-glycosidic branches, but with branches arranged in a discontinuous pattern every 12–20 glucose residues (Table 1). This decreased amount of branching renders amylopectin, and thus the starch, insoluble. While LBs are often referred to as "insoluble glycogen," the definitive biochemical studies on LBs found that they most closely resemble "amylopectin and plant glycogen," i.e., starch, and not true glycogen (Sakai et al., 1970; Yokoi et al., 1967, 1968).

Starch-like polymers are only produced in photosynthetic eukaryotes and their nonphotosynthetic derivatives, such as some members of apicomplexa (*Toxoplasma gondii* and *Eimeria tenella*), ciliaphora (*Tetrahymena thermophila* and *Paramecium tetraurelia*), and dinoflagellata (Myers et al., 2000). Green algae and higher plants produce starch from ADPglucose inside the plastids. In contrast, nonphotosynthetic organisms and photosynthetic red algae produce floridean starch in the cytoplasm by utilizing UDPglucose (Coppin et al., 2005; Guérardel et al., 2005; Meeuse et al., 1960; Nyvall et al., 1999; Viola et al., 2001). The structure of floridean starch and starch are quite similar even though the sugar nucleotides are different (Myers et al., 2000) (Table 1). The main differences between the two molecules are that the starch contains more amylose than floridean starch and is produced within the plastid, while floridean starch is synthesized in the cytoplasm.

3. Discovery of Laforin in Protists

One protist that generates floridean starch (also called amylopectin granules) in its cytoplasm is *Toxoplasma gondii* (Coppin et al., 2003, 2005; Dubey and Frenkel, 1972; Guérardel et al., 2005). *Toxoplasma gondii* is an obligate intracellular parasite that can infect virtually any nucleated cell, and like most other Apicomplexa, has a complex life cycle. Cats are the definitive host and the only species in which the sexual phase of the life cycle can be propagated. In intermediate hosts, *T. gondii* exists as a rapidly dividing tachyzoite until the host's immune system detects it and responds to the invader. Upon detection, the tachyzoite transitions into an encysted bradyzoite within a variety of tissues to escape detection by the host's immune system. The bradyzoite produces floridean starch in its cytoplasm and uses this product as an energy source (for review see Coppin et al., 2003).

Recent studies characterized the biochemical composition of *T. gondii* floridean starch (Coppin et al., 2005; Guérardel et al., 2005). We noted that the biochemical composition of *T. gondii* floridean starch is strikingly similar to that of LBs described nearly 40 years ago (Sakai et al., 1970; Yokoi et al., 1967, 1968). Thus, we surmised that starch, floridean starch, and LBs are quite similar with respect to glucose residues per branch, branching pattern, and insolubility (Table 1), and postulated that any organism that generates these carbohydrates would possess a laforin-like protein.

Although the gene encoding laforin was reported to be present only in vertebrates (Ganesh et al., 2001, 2004), the similarity between *T. gondii* floridean starch and LBs prompted us to search the partially completed *T. gondii* genome for a laforin ortholog (Gentry et al., 2007). We used the criteria that a laforin ortholog must contain an amino-terminal CBM and a carboxy-terminal DSP (Fig. 1b). CBMs are very degenerate at the primary amino acid level and are not readily recognized either by the protein families database (pfam; Bateman et al., 2004) or by the National Center for Biotechnology Information's (NCBI) conserved domain database (CDD; Marchler-Bauer et al., 2005). Therefore, we devised a multitiered strategy to search ToxoDB (Fig. 2) (Gentry et al., 2007). We used the DSP motif of laforin, HCXXGXXR, as the index sequence, performed BLAST searches, identified all DSPs that contained at least 100 amino acids amino-terminal of the DSP domain (the size and location of Hs-laforin's CBM), and then performed a secondary BLAST with the identified protein minus its DSP domain (Fig. 2). If the identified protein contained a CBM, then the secondary BLAST identified glycosylhydrolases and glucotransferases (which contain CBMs), according to our prediction, and we identified one putative ortholog (Gentry et al., 2007).

With the discovery of a putative *T. gondii* laforin ortholog, we extended our search strategy to additional genome databases (Gentry et al., 2007). We identified

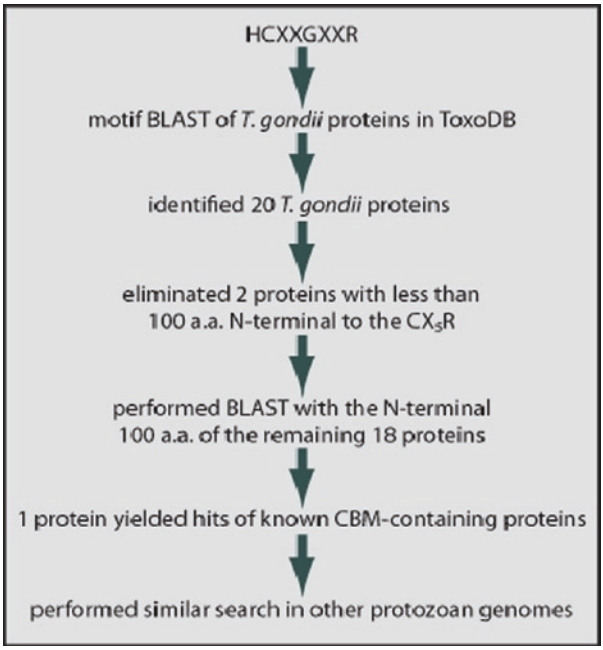

Figure 2. Strategy to identify laforin orthologs in protozoan genomes.

putative laforin orthologs in four additional protozoan genomes: *Eimeria tenella*, *Tetrahymena thermophila*, *Paramecium tetraurelia*, and the red alga *Cyanidioschyzon merolae*. The degree of similarity between *Homo sapiens* laforin (Hs-laforin) and the protozoan orthologs varied from 35% to 51%, but all putative orthologs contained the signature amino acids of both CBM20 and DSP (Gentry et al., 2007). In total, we searched ~170 eukaryotic genomes and ~670 bacterial and archaeal genomes, but did not identify any other putative laforin orthologs. Therefore, laforin is absent in the sequenced genomes of all traditional invertebrate and protist model organisms (e.g., fly, worms, and yeast), and is only found in vertebrates and these five protists.

4. Biochemical Properties and Subcellular Localization of *C. merolae* Laforin

Out of the five putative protozoan laforin orthologs, *C. merolae* laforin (Cm-laforin) shares the least similarity (35%) with Hs-laforin (Gentry et al., 2007). We reasoned that if Cm-laforin exhibited *in vitro* characteristics similar to those of Hs-laforin, the other putative orthologs were likely to exhibit those as well. Characteristic of all DSPs, Hs-laforin exhibits phosphatase activity against the artificial substrate *para*-nitrophenylphosphate (*p*-NPP) (Ganesh et al., 2000). Additionally, laforin is the only human phosphatase shown or predicted to bind to complex carbohydrates, e.g., glycogen, starch, and amylopectin (Wang et al., 2002). Furthermore, laforin is the only phosphatase that has been shown to release phosphate from amylopectin (Worby et al., 2006). Therefore, if a phosphatase is an ortholog of laforin, it should possess the following characteristics: (1) exhibit phosphatase activity against *p*-NPP, (2) bind to complex carbohydrates, and (3) dephosphorylate amylopectin. To test the biochemical properties of Cm-laforin, we cloned the gene from *C. merolae* genomic DNA, expressed Cm-laforin in bacteria, and purified it to near homogeneity (Gentry et al., 2007).

Similar to Hs-laforin, Cm-laforin exhibited phosphatase activity against *p*-NPP, bound complex carbohydrates, and released phosphate from amylopectin (Gentry et al., 2007). In addition, the *T. gondii* putative laforin ortholog (Tg-laforin) also possessed these characteristics (Gentry et al., 2007). Therefore, Cm-laforin is an ortholog of Hs-laforin, similar to the other four putative protozoan laforin orthologs.

While all three orthologs of laforin were observed to bind to α-glucans *in vitro*, the localization of endogenous laforin had never been described in any wild-type organism. *Cyanidioschyzon merolae* typically generates floridean starch during periods of light, and utilizes this carbon source to perform dark reactions during periods of darkness. When grown in continuous light, *C. merolae* generate vast stores of floridean starch (Viola et al., 2001). Using a polyclonal antibody that recognized Cm-laforin, we demonstrated that Cm-laforin is found exclusively in the cytoplasm of *C. merolae* decorating the floridean starch (Gentry et al., 2007).

5. Laforin's Evolutionary Lineage

As previously stated, we identified putative laforin orthologs in five protozoan genomes: *Toxoplasma gondii*, *Eimeria tenella*, *Tetrahymena thermophila*, *Paramecium tetraurelia*, and the red alga *Cyanidioschyzon merolae* (Gentry et al., 2007). In addition to these protists, laforin was found to be conserved in all five classes of vertebrates. Besides these five protists and all vertebrates, no other sequenced genome was observed to contain a laforin ortholog. Thus, laforin is not conserved in yeast, flies, worms, or any other organism with a sequenced genome. The question then arises: why is laforin conserved in vertebrates and these five protists? The answer lies in the chromalveolate hypothesis (Cavalier-Smith, 1999).

As per the chromalveolate hypothesis (Cavalier-Smith, 1999), a mitochondriate protist engulfed a cyanobacterium, gene transfer occurred between the two, eventually, the cyanobacterium was reduced to a plastid within the protist, and the plastid was transmitted to subsequent generations (Bhattacharya and Medlin, 1998; Cavalier-Smith, 1982) (Fig. 3a). This initial engulfment gave rise to three offsprings (glaucophytes, green algae/land plants, and red algae) that are the basis of Kingdom Plantae (Cavalier-Smith, 2004) (Fig. 3a). Once Plantae was established, a secondary endosymbiosis occurred, whereby a protist engulfed a red algae (RA) (Gillott and Gibbs, 1980) (Fig. 3b). This secondary endosymbiosis gave rise to the chromalveolates, encompassing Kingdom Chromista, the apicomplexa (including *T. gondii* and *E. tenella*), ciliates (including *T. thermophila* and *P. tetraurelia*), and some dinoflagellates (Cavalier-Smith, 1999). Intermingled with these engulfments and gene transfers was the evolution of "various manifestations of mitochondria" (Embley and Martin, 2006) and the diverse forms of carbohydrate storage (Viola et al., 2001). These combined evolutionary events resulted in organisms possessing a mitosome, a hydrogenosome, or a mitochondrion; and some organisms evolved floridean starch as their storage carbohydrate. We hypothesized that interspersed with these evolutionary events, organisms lost, maintained, or gained laforin.

To outline the evolutionary lineage of laforin, we generated a phylogeny derived from the small subunit (SSU) ribosomal RNA (rRNA) gene of organisms belonging to separate evolutionary niches, and highlighted the organisms whose genome contains laforin (Fig. 3c) (Gentry et al., 2007). Each of the organisms that contained laforin were of red alga descent, contained a true mitochondrion, and produced floridean starch (Gentry et al., 2007). Conversely, an organism lacking any of these three criteria also lacked laforin. These three criteria held true for all 168 eukaryotic organisms and genomes that we investigated (Gentry et al., 2007). Therefore, based on these three criteria one can predict if the genome of an organism contains laforin.

From this work, we hypothesize that in protists, laforin is involved in the metabolism of floridean starch. Additionally, as vertebrates do not normally synthesize insoluble carbohydrates, this suggests that laforin inhibits insoluble carbohydrate formation in vertebrates and is involved in the catabolism of floridean starch in protists.

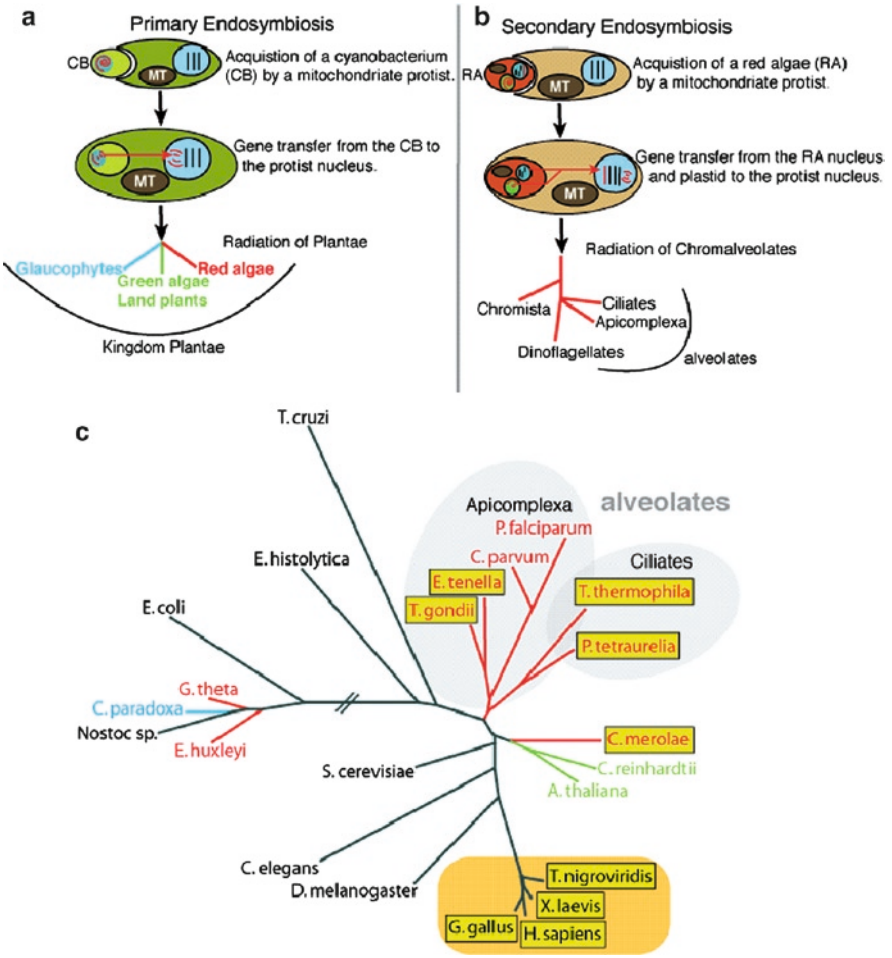

Figure 3. Evolutionary lineage of laforin and malin. (**a**) Primary endosymbiosis hypothesized by the chromalveolate hypothesis (Cavalier-Smith, 1999). A cyanobacterium (CB) was engulfed by a mitochondriate protist (Bhattacharya and Medlin, 1998; Cavalier-Smith, 1982) (MT, mitochondrion). Over generations, gene transfer occurred between the engulfed CB and protist, the CB was reduced to a plastid bound by two membranes, and the plastid containing protist radiated into the founding members of Kingdom Plantae (Cavalier-Smith, 2004) (reproduced from Gentry et al., 2007). (**b**) Secondary endosymbiosis hypothesized by the chromalveolate hypothesis (Cavalier-Smith, 1999). A red alga (RA) was engulfed by a mitochondriate protist (Gillott and Gibbs, 1980). Over generations, gene transfer occurred from the RA nucleus and plastid to the nucleus of the protist, the RA was reduced to a plastid bound by three or four membranes, and the new protist radiated into Kingdom Chromista and the alveolates, collectively the chromalveolates (Cavalier-Smith, 1999). (**c**) Conservation of laforin and malin orthologs. A phylogeny of the small subunit (SSU) ribosomal RNA (rRNA) sequences was generated. Organisms containing laforin are boxed in yellow. Organisms from green algal descent are in green, organisms from glaucophyte descent are in blue, and organisms from red algal descent are in red. Alveolates are shaded with a gray background. Organisms containing malin are shaded with an orange background, and these are all vertebrates.

6. Lack of Malin

While laforin is conserved in vertebrates and in a subclass of protists, malin is conserved only among vertebrates (Fig. 3c) and is not present in any lower eukaryotes, protists, or plants. We employed a search strategy similar to the strategy devised for laforin orthologs (Fig. 2), but our efforts to identify malin orthologs in nonvertebrate species failed. In addition, we searched for malin-like proteins containing a RING domain linked to NHL domains in any orientation, and these too failed to produce any malin orthologs in organisms other than vertebrates.

This result is surprising, given that malin ubiquitinates laforin and regulates the abundance of laforin in human tissue culture models (Gentry et al., 2005). Furthermore, several studies have definitively established that laforin and malin function together during glycogen metabolism (Gentry et al., 2007, 2005; Solaz-Fuster et al., 2008; Vilchez et al., 2007; Wang et al., 2006; Worby et al., 2006, 2008). Thus, malin and laforin appear to function collectively to regulate carbohydrate metabolism in vertebrates, but malin is absent in nonvertebrates that contain laforin.

Though surprising, several lines of reasoning can explain this observation, and shed further light on the evolutionary lineage of malin and laforin. The simplest explanation is that lower organisms that contain laforin (i.e., the five protists) and are missing a canonical malin or malin-like protein contain another E3 ubiquitin ligase that can function as malin. These organisms either contained such an E3 enzyme at the onset of their conception or acquired it during evolution. Importantly, the five protists that contain laforin and lack malin do contain all of the components of the ubiquitination pathway. Thus, the lack of malin is not merely owing to the fact that organisms lack the entire ubiquitination system.

Alternative possibilities are that the protozoan organisms regulate laforin either by a nonubiquitin post-translational modification or by controlling laforin mRNA levels. Regulating the mRNA abundance and half-life is a common theme in starch metabolism in plants. Plants synthesize starch and shuttle it for storage to two locations: (1) starch in the chloroplasts of leaves accumulates over short periods during light and is remobilized as an energy source each night, following a diurnal pattern, and (2) starch in the amyloplasts of storage organs accumulates over a long developmental period and is not utilized on a daily basis. The mRNA levels of the enzymes involved in starch synthesis largely remain unchanged during the diurnal cycle; however, the mRNA of many enzymes involved in starch catabolism exhibit a coordinated rapid accumulation toward the end of the light phase and decline during the dark phase (Smith et al., 2004; Yu et al., 2005; Zeeman et al., 2002). Similarly, one could envision that protozoans regulate the mRNA of laforin in a similar manner. On the other hand, vertebrates regulate laforin protein abundance via malin and the ubiquitin proteasome pathway.

It is intriguing that laforin and malin have converged on a common function (carbohydrate metabolism) without having co-evolved. Malin appears solely in vertebrates, while laforin appears periodically throughout the tree of life. Laforin is present in some apicomplexa, some ciliates, some metazoans, some glaucophytes, and in

vertebrates, but is absent in nonvertebrate species, such as *S. cerevisiae*, *C. elegans*, and *D. melanogaster* (Fig. 3c). It is unusual for a protein to skip the entire clades of evolutionary lineages, appearing intermittently among lower organisms, and then be very highly conserved among all vertebrates. We, therefore, propose that laforin was present early in the tree of life (according to the chromalveolate hypothesis, see Fig. 3); it was then lost in those organisms where its role became vestigial, but was retained by organisms that adapted to regulate carbohydrate metabolism. Conversely, malin might have been absent initially in the tree of life, and was probably acquired in a higher organism(s) by horizontal gene transfer or other means.

7. Insights from Green Algae/Land Plants

Protists such as *T. gondii* and *E. tenella* utilize insoluble carbohydrates as an energy source when (1) transitioning from inactive/hibernating lifecycle stages to active/replicating stages or (2) as an energy source during a hibernating state. *Cyanidioschyzon merolae* experiences an energy-poor, "hibernating" state every 24 h, whenever sunlight is not present. Plants undergo a similar diurnal cycle, and produce insoluble carbohydrate in the form of starch during the day and catabolize it during the night.

Several mutants have recently been described that affect starch metabolism and cause an accumulation of starch in plant leaves (Blennow et al., 2002; Smith et al., 2005; Zeeman et al., 2007). One of these mutants is in the s*tarch* e*xcess 4* (*SEX4*) gene (At3g52180, previously identified as *AtPTPKIS1* and also called *DSP4*) (Fordham-Skelton et al., 2002; Niittyla et al., 2006; Sokolov et al., 2006). *Arabidopsis thaliana* plants mutated in *SEX4* accumulate an excess amount of starch, because they cannot catabolize starch in a wild-type manner (Niittyla et al., 2006). Additionally, two groups recently showed that the SEX4 protein contains a DSP domain followed by a CBM (Fig. 1c) (Kerk et al., 2006; Niittyla et al., 2006), with domains similar to those of laforin but in the opposite orientation. The identification of a second phosphatase containing a CBM strongly suggests that SEX4 might be a laforin-like phosphatase (Niittyla et al., 2006).

The DSP of SEX4 is very similar to the DSP of Hs-laforin (39% similar) (Gentry et al., 2007). In addition, SEX4 can utilize *p*-NPP as an artificial substrate and can release phosphate from amylopectin (Gentry et al., 2007). The CBM of SEX4 is similar to the CBM of laforin, but is classified as a different subclass of CBM (AMPKβ-GBD versus CBM20, respectively). Also, similar to laforin, SEX4 binds to complex carbohydrates *in vitro* (Gentry et al., 2007). Thus, it can be concluded that SEX4 possesses domains and biochemical properties similar to laforin, but the domains are in the opposite orientation (Fig. 1b versus Fig. 1c).

Unlike laforin, SEX4 is conserved in all land plants and green algae investigated to date, from multi-cellular *Arabidopsis thaliana* to the single-celled green alga *Chlamydomonas reinhardtii* (Gentry et al., 2007). Therefore, it is likely that SEX4 evolved before or during the establishment of green algae and performs a

conserved function in Kingdom Plantae. Additionally, as mutations in laforin or SEX4 result in the accumulation of insoluble carbohydrates, it is likely that they both participate in carbohydrate metabolism. As carbohydrate metabolism evolved independently in Kingdom Plantae and Kingdom Animalia, the use of similar protein modules to regulate insoluble carbohydrate metabolism is an elegant example of convergent evolution.

In addition to being an example of convergent evolution, these similarities also suggest that laforin and SEX4 could be functional equivalents, i.e., two different proteins that perform the same function in different species. We tested this hypothesis by expressing Hs-laforin in *sex4*-mutant *Arabidopsis* plants. We found that *sex4*-mutant plants stably transfected with Hs-laforin accumulate starch similar to that by wild-type plants (Gentry et al., 2007). Thus, Hs-laforin rescues the *sex4*-mutant phenotype, and laforin and SEX4 are functional equivalents.

It should be stated that although laforin and SEX4 share a common function and similar domains, they are not orthologous proteins. They are not orthologs because (1) their CBMs differ considerably at the primary amino acid level, and hence, are classified as different types of CBMs, and (2) the DSP and CBM domains of laforin and SEX4 are in opposite orientations (Fig. 1b versus Fig. 1c). Therefore, it is probable that red and green algae independently evolved a phosphatase (laforin and SEX4, respectively), via convergent evolution, that can regulate insoluble carbohydrate metabolism.

8. The Role of Phosphorylation in Starch Metabolism

We demonstrated that laforin and SEX4 could remove phosphate from amylopectin (Gentry et al., 2007). Furthermore, we showed that this was a unique activity to laforin and SEX4; therefore, laforin and SEX4 form a novel class of phosphatases that remove phosphate from complex carbohydrates. Finally, we postulated that laforin dephosphorylates complex carbohydrates to inhibit insoluble carbohydrate synthesis in vertebrates, and laforin and SEX4 regulate insoluble carbohydrate catabolism in protists and plants, respectively. However, two questions arise from these results and hypotheses. How does phosphate get incorporated into starch? Where is phosphate located within starch, i.e., which of the six positions of glucose gets phosphorylated?

While starch is degraded by enzymes that cleave α-1,4 or α-1,6 glucosidic linkages, enzymes that phosphorylate starch are also necessary for normal starch degradation in leaves (Blennow et al., 2002; Smith et al., 2005; Zeeman et al., 2007). Glucan water dikinase (GWD; also called R1 and SEX1) specifically adds a monophosphate to the C6 position of glucan residues within starch (Ritte et al., 2006, 2002). Once the C6 position is phosphorylated, C3 monophosphate esters are catalyzed by phosphoglucan water dikinase (PWD) (Baunsgaard et al., 2005; Kotting et al., 2005; Ritte et al., 2006). These kinases utilize a unique mechanism amongst the kinase super family by transferring the β-phosphate of ATP to the glucan

(Kotting et al., 2005; Ritte et al., 2002). Mutations in either *GWD* or *PWD* result in plants that accumulate an excess amount of starch in their leaves (Baunsgaard et al., 2005; Kotting et al., 2005; Yu et al., 2001), similar to those with mutations in *SEX4*. While phosphorylation of starch is clearly necessary for both starch accumulation and degradation, the timing of these phosphorylation and dephosphorylation events are currently unknown (Smith et al., 2005; Zeeman et al., 2007).

Strikingly, while glycogen contains low levels of monophosphate (Lomako et al., 1994, 1993), detrimental insoluble carbohydrates like LBs are highly phosphorylated (Sakai et al., 1970; Schnabel and Seitelberger, 1968), just like amylopectin in plant starch (Blennow et al., 2002) (Table 1). Therefore, it is likely that laforin and SEX4 evolved to remove phosphate from insoluble carbohydrates to inhibit their accumulation in vertebrates and promote their degradation to release energy stores in protists and plants.

By modifying a model proposed by Gerhard Ritte et al., (Edner et al., 2007), one could envision that phosphate is added to amylopectin helices in the outer layer of starch (Fig. 4). This phosphorylation would cause the helices to unwind and make the outer layer more water-soluble. The increased solubility of these helices would make them more accessible to carbohydrate-metabolizing enzymes, like β-amylase. Once the degradation enzymes have acted, phosphate at the base of the helical glucan may inhibit further degradation from progressing; therefore, the phosphate must be removed by SEX4. This process would then be repeated on subsequent starch layers to promote degradation. Therefore, the phosphate acts as both an activator and repressor of degradation. The glucan must be phosphorylated to be degraded, but the phosphate must be removed for the subsequent layer to be degraded. This dual role of activator and repressor is reminiscent of cell-cycle proteins that promote passage into one phase of the cell cycle, but then must be degraded before entry into the next phase. This duplicity would allow cells a mechanism to intricately manipulate their starch stores and only degrade what is needed.

There is no indication that glycogen is phosphorylated and dephosphorylated in a similar manner during its metabolism. However, one could envision that laforin ensures that glycogen is kept in a dephosphorylated state to ensure that LBs do not

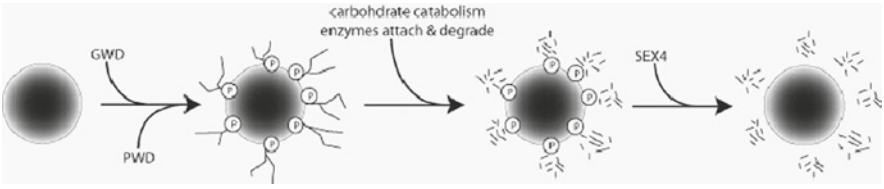

Figure 4. Proposed model of the role of phosphorylation and laforin in carbohydrate metabolism. Starch is phosphorylated by GWD and then by PWD. Phosphorylation makes the outermost layer accessible to the enzymes that degrade starch. After degradation, laforin removes the phosphate and this allows the next round of degradation to proceed.

form. Monophosphate could be incorporated into glycogen during synthesis, as a remnant of evolution, or a kinase could indiscriminately add phosphate to glycogen. Along these lines, there are multiple kinases that bind glycogen, e.g., hexokinase/glucokinase and AMP-dependent protein kinase (AMPK), and laforin could act as a general default mechanism to inhibit one of these kinases.

9. *Cyanidioschyzon merolae* as a Model Organism to Study Lafora Disease

Two transgenic mouse models have been developed for LD. Ganesh et al. (2002) disrupted the *EPM2A* gene to generate null mice, and Chan et al. (2004) generated transgenic mice by over-expressing inactivated laforin, laforin^{C266S}, in all tissues. The mouse models mimicked the human disease to different degrees, but neither mouse model yielded abundant molecular insights into the role of laforin in LD. Therefore, we have proposed to employ *C. merolae* as a model organism to study LD and have taken the advantage of the genetic, biochemical, and cell biology tools available in *C. merolae*.

Cyanidioschyzon merolae has been utilized by Dr. Tsuneyoshi Kuroiwa's team to decipher chloroplast and mitochondrial division (Miyagishima et al., 1999, 2001; Nishida et al., 2003, 2005). With the completion of its genome, *C. merolae* has been the recent focus of multiple comparative genomic studies (Barbier et al., 2005; Matsuzaki et al., 2004; Misumi et al., 2005, 2008). Dr. Kuroiwa's team pioneered a variety of cell-biology techniques using *C. merolae*, including protein isolation, Western analysis, cell synchronization, immunofluorescence, transmission electron microscopy, and immunoelectron microscopy (Matsuzaki et al., 2004; Miyagishima et al., 1998, 2001, 2003; Nishida et al., 2003, 2005). Proteins are easily isolated by boiling cells in SDS-PAGE buffer (Takahara et al., 2000) and RNA can be isolated using TRIzol (Invitrogen) (Ohta et al., 1994). In addition, many chemicals readily cross *C. merolae*'s cell wall, including aphidicolin (a DNA polymerase inhibitor), 5-fluorodeoxyuridine (5FdU, which arrests cells in S-phase), MitoTracker Red (a mitochondrial dye), oryzalin and trifluralin (plant and algal microtubule antagonists), and MG132 (a proteasome inhibitor) (Itoh et al., 1996; Miyagishima et al., 1999; Nishida et al., 2005). Many cell-cycle manipulations have been performed using *C. merolae,* and its mitotic division has been well documented (Misumi et al., 2005).

Red algae, like *C. merolae*, synthesize floridean starch during times of darkness, and culturing conditions to modulate floridean starch synthesis and catabolism are well documented and are nearly as simple as those for *S. cerevisiae* (Allen, 1959; Ekman et al., 1991; Nyvall et al., 1999; Sesma, 1998). *Cyanidioschyzon merolae* is grown by shaking at 225 rpm in 2× Allen's medium of pH 2.4 with continuous 80 mmol photons m^{-2} s^{-1} at 42°C. The cells can be synchronized by subjecting them to a 12-h light/12-h dark diurnal regimen with 5% CO_2 aeration; the synchronization option allows for detailed cell-cycle analysis (Allen, 1959; Minoda et al., 2004; Miyagishima et al., 1999; Nishida et al., 2003, 2005). Recent reports have described successful transformation of DNA into the nucleus of *C. merolae*,

defined selectable markers, and showed that homologous recombination occurs, suggesting that stable gene knock-outs should soon be possible (Minoda et al., 2004; Ohnuma et al., 2008; Yagisawa et al., 2004). Notably, *C. merolae* has a haploid genome, thus genetic manipulation should be relatively simple.

These techniques and characteristics offer great advantages in studying the control of insoluble carbohydrate metabolism in a genetically manipulatable organism. Understanding the basic controls of floridean starch metabolism in *C. merolae* may elucidate the molecular mechanisms that drive LD. In addition, *C. merolae* could be utilized in screens to test inhibitors and/or activators of floridean starch metabolism. These results could be translated to mouse studies for better understanding of the mechanisms of LD and to uncover a potential therapeutic agent. These findings and thoughts demonstrate that "domains that are far apart" are often "the most fertile."

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COORDINATION OF NUCLEAR AND PLASTID GENE EXPRESSION IN RED ALGAE AND GREEN PLANTS

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1. Introduction

Chloroplasts of plant cells are semiautonomous, photosynthetic organelles, which are thought to have originated from endosymbiosis of an ancient cyanobacterium (Lopez-Garcia and Moreira, 1999). As one of the major relics of this event, chloroplasts have their own genome and genetic systems similar to those in cyanobacteria. However, during the course of subsequent evolution, most of the bacteria-derived genes were lost, while a number of genes were transferred into and included in the nuclear genome of the host eukaryotic cell (Martin, 2003; Timmis et al., 2004). Consequently, genes required for normal plastid function have been encoded separately on nuclear and plastid genomes. This indicates that some signaling mechanisms are essential to regulate cooperative expression between the two different genetic systems (Goldschmidt-Clermont, 1998). Apparently, various regulatory systems operating many chloroplast functions, such as photosynthesis and metabolism, were posed under the control of the nucleus, accompanied by the loss of genes from chloroplast genomes. On the other hand, many lines of evidence indicate the presence of retrograde signaling mechanisms that convey chloroplast information into nuclear gene expression.

In this chapter, we focus on recent advances in the fields of regulation of chloroplast gene expression and of communication between nucleus and chloroplasts in red algae and green plants, including comparison of both systems. In particular, we introduce and discuss the systems in *Cyanidioschyzon merolae* and *Arabidopsis thaliana*, the model organisms of red algae and green plants in the postgenomic age. In both these organisms, the three kinds of genomic DNA sequences (nucleus, mitochondria, and chloroplasts) have been completely sequenced.

2. Plastid Genome of Red Algae and Green Plants

Based on the morphology and the type of photosynthetic pigments of chloroplasts, most photosynthetic eukaryotes belong to one of the three major evolutionary groups: Rhodophyta, comprising several algal groups; Glaucophyta, such

as *Cyanophora paradoxa* and Chlorophyta, which comprises green algae and land plants (McFadden, 2001). Chloroplast genomes in each group were found to be derived from the common endosymbiosis of ancestral cyanobacteria, and are composed of a double-stranded, circular DNA molecule ranging from 100 to 250 kbp in size. However, the number and content of their genes are rather different from each other (Martin et al., 1998).

Historically, the DNA molecule was biochemically detected in *Chlamydomonas reinhardtii* chloroplasts in the early 1960s (Sager and Ishida, 1963). After further mapping analyses, the complete DNA sequences of the chloroplast genome were first reported in *Marchantia polymorpha* (Ohyama et al., 1986) and *Nicotiana tabacum* (Shinozaki et al., 1986). During the subsequent 20 years, sequencing of the entire promoter elements, respectively (reviewed in Allison, 2000; Lysenko, 2007).

During the subsequent 20 years, sequencing of the entire chloroplast genome was then extended to red algae such as *Porphyra purpurea* (Reith and Munholland, 1995), *Cyanidium caldarium* (Glöckner et al., 2000), and *C. merolae* (Ohta et al., 2003), as well as to green plants including *Oryza sativa* (Hiratsuka et al., 1989), *Triticum aestivum* L (Ogihara et al., 2000), and *A. thaliana* (Sato et al., 1999).

In the case of green plants, the gene content of the chloroplast genome does not vary greatly from species to species, and comprises about 100–120 genes (Sugita and Sugiura, 1996). In *A. thaliana*, chloroplast genes have been identified as encoding 4 rRNAs, 30 tRNAs, and 79 proteins, all of which are required for transcription, translation, and chloroplast functions such as photosynthesis (Sato et al., 1999). As for the transcription apparatus, genes for the catalytic core subunits of the RNA polymerase (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*) are generally located on chloroplast DNA, and those for the other regulatory components were completely lost from the chloroplast genome. The only exceptional case is reported in *Physcomitrella patens*, in which two copies of the *rpoA* gene are encoded by the nuclear genome (Kabeya et al., 2007).

On the other hand, the gene content of the chloroplast genome in red algae varies significantly depending on the organism. The total gene number on the chloroplast genome of *P. purpurea*, a multicellular red alga, or *C. caldarium* and *C. merolae*, unicellular red algae, comprises ~250 genes, which is approximately twice that of green plants. Approximately half of these are genes that are encoded on the chloroplast genome in green plants, which are mostly conserved on the red algal chloroplast genome as well. However, the content of the other half of the genes varies depending on the organism. For example, *C. caldarium* lacks 39 genes present in *P. purpurea* while *C. caldarium* possesses 28 genes that are not conserved in *P. purpurea* (Glöckner et al. 2000). Both *C. caldarium* and *C. merolae* are unicellular red algae living in an extremely restricted environment—acidic hot springs. Their chloroplast genomes are thus assumed to be well conserved during the long history of chloroplast evolution, and most genes are found in both organisms. However, some genes are unique to one or the other; for example, *cysW*, a gene for a subunit of sulfate-transporter, is unique to *C. merolae* (Ohta et al., 2003). Furthermore, the red lineage includes a group of diatoms or cryptophytes such as

Odontella sinensis and *Guillardia theta*, the chloroplasts of which were derived from a secondary endosymbiotic event (Gibbs, 1981). In these algae, gene content and organization of the chloroplast genome were significantly diverged from other members of the red algae, due to a major displacement of the chloroplast genome (Douglas, 1998; Douglas and Penny, 1999; Kowallik et al., 1995).

3. Plastid RNA Polymerase

According to the structural similarity of chloroplast genomes to bacterial ones, as well as the strong conservation of genes encoding core subunits for the plastid RNA polymerase (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*) compared with those of bacteria, transcription of chloroplast genes is assumed to be mediated by a bacteria-type RNA polymerase. In the chloroplast of higher plants, however, at least two distinct RNA polymerases, named NEP (Nuclear-encoded plastid RNA polymerase) and PEP (Plastid-encoded plastid RNA polymerase), were found to be involved in transcription of chloroplast genes (Figure 1), indicating that the genetic system in chloroplasts is more complex than expected (reviewed in Shiina et al., 2005). In this section, we briefly introduce characteristics of the two different forms of plastid RNA polymerases, and their roles in red algae and green plants.

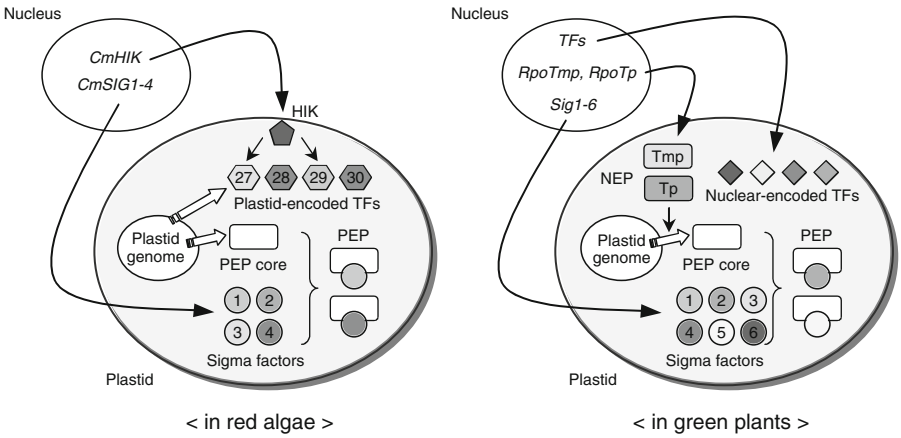

Figure 1. Plastid transcription machineries in red algae (*left*) and green plants (*right*). In red algae (*C. merolae*), plastid genes are transcribed by PEP assembled with one of four nuclear-encoded sigma factors. Transcription regulation is also mediated by four plastid-encoded transcription factors (TFs; Ycf27–30). Among these, Ycf27 and Ycf29 are response regulators whose activity can be regulated by the histidine kinase (HIK). In green plants (*A. thaliana*), PEP with six sigma factors also functions in chloroplast transcription. In addition, another type of RNA polymerase (NEP; RpoTmp and RpoTp) and some nuclear-encoded transcription factors are involved in transcription regulation in chloroplasts.

3.1. PEP (PLASTID-ENCODED PLASTID RNA POLYMERASE)

PEP is a bacteria-type, multisubunit enzyme which is composed of a, b, b', b" and w subunits encoded by *rpoA*, *rpoB*, *rpoC1*, r*poC2* and *rpoZ* chloroplast genes, respectively. Since a typical bacterial RNA polymerase consists of a, b, b' and w subunits, one of the major structural differences from a bacterial RNA polymerase is the split of the b' subunit into b' and b" subunits, except for the RNA polymerase in *C. merolae*, which consists of a, b, b' and w subunits (Ohta et al., 2003). However, the RNA polymerase in cyanobacteria is composed of a, b, g, b' and w subunits (g and b' subunits of cyanobacterial RNA polymerase are homologous to b' and b" subunits, respectively, of the chloroplast RNA polymerase), which also supports the structural similarity of RNA polymerases between cyanobacteria and chloroplasts. Thus, although the w subunit in green plants has not yet been identified, the function of these types of RNA polymerases appears to be similar.

PEP is mainly responsible for the transcription of photosynthesis genes, which has been established from several genetic analyses (De Santis-Maciossek et al., 1999; Hajdukiewicz et al., 1997; Legen et al., 2002). In the upstream region of many photosynthesis genes, bacterial-type –35 (TTGACA) and –10 (TATAAT)-like promoter elements are well conserved. The importance of these elements in PEP-dependent transcription was also characterized *in vitro* (Kim et al., 1999; Satoh et al., 1999) and *in vivo* (Hayashi et al., 2003; Thum et al., 2001). These promoter elements are recognized by one of many sigma factors that are involved in transcription regulation of chloroplast genes (discussed below). In principle, PEP has been widely conserved in every chloroplast lineage, suggesting that PEP was predominantly involved in transcription in common ancestral chloroplasts.

3.2. PEP SIGMA FACTORS

A sigma factor, one of the subunits of the bacterial RNA polymerase, binds to the catalytic core enzyme to form a holo-RNA polymerase that can recognize specific promoter elements and initiate transcription. In general, there are multiple sigma factors in each organism, which have different specificities of promoter recognition, indicating that selective transcription regulation can be achieved by differential use of sigma factors in response to developmental and environmental conditions. In the case of the bacteria-type RNA polymerase (PEP) in chloroplasts, genes for subunits of the core enzyme (*rpoA*, *rpoB*, *rpoC1*, and r*poC2*) are located on the chloroplast genome as described above. In contrast, those encoding sigma factors are not found on the chloroplast genome while they could be detected biochemically (Tiller et al., 1991) or immunologically (Troxler et al., 1994), suggesting that sigma factors are encoded by the nuclear genome.

In 1996, nuclear genes for PEP sigma factors were isolated from two unicellular red algae, *C. caldarium* RK1 (Tanaka et al., 1996) and *Galdieria sulphuraria* (Liu and Troxler, 1996). Their multiplicity in *C. caldarium* was then established

(Oikawa et al., 1998). Genome analysis in *C. merolae* revealed four sigma factor genes on its nuclear genome (Matsuzaki et al., 2004). In green plants, six nuclear genes encoding chloroplast sigma factors were identified in *A. thaliana* (Isono et al., 1997; Tanaka et al., 1997; Fujiwara et al., 2000), followed by cloning from many flowering plants including rice (Tozawa et al., 1998) and tobacco (Oikawa et al., 2000), a moss, *P. patens* (Hara et al., 2001; Ichikawa et al., 2004), as well as from a green alga, *C. reinhardtii* (Carter et al., 2004; Bohne et al., 2006). Given that only one sigma factor is encoded on the *C. reinhardtii* nuclear genome, it can be speculated to have an essential function.

Chloroplast sigma factors in both red algae and green plants shared high similarity to those in bacteria in their conserved regions, including subregions 2.4 and 4.2, which are required for recognition of –10 and –35 promoter elements, respectively (reviewed in Allison, 2000; Lysenko, 2007).

In addition, all of them have extra sequences at their N-terminus, functioning as transit peptides for proper import into chloroplasts. It has been established that some plastid sigma factors actually bind to PEP promoter sequences and initiate accurate transcription *in vitro* (Hakimi et al., 2000; Hanaoka et al., 2003; Homann and Link, 2003).

According to analyses of exon–intron structures, transfer of sigma factor genes into the nuclear genome is thought to have occurred at a very early stage after endosymbiosis, and was followed by gene duplication within the nuclear genome (Lysenko, 2006). The *Sig5* gene in land plants is rather exceptional, and may have been transferred independently from other sigma factor genes, or may have diverged from an ancestral nuclear gene at an early stage of evolution.

3.3. NEP (NUCLEAR-ENCODED PLASTID RNA POLYMERASE)

Other than PEP, a 110 kDa protein in spinach was suggested to function as another RNA polymerase by biochemical fractionation of purified chloroplast proteins (Lerbs-Mache, 1993). In addition, genetic analysis using the tobacco PEP-deficient mutant revealed that transcription of some genes was still active in the absence of PEP (Hajdukiewicz et al., 1997). These observations supported the idea that another, nuclear-encoded plastid RNA polymerase (NEP) could function in chloroplasts. Subsequently, two nuclear genes encoding single-subunit RNA polymerases, which were imported into mitochondria and chloroplasts, respectively, were isolated from *A. thaliana* (Hedtke et al., 1997). This RNA polymerase shares homology with those of T7-, T3-, or SP6-type bacteriophages, and structure of the functional domains required for catalytic activity was highly conserved among divergent mitochondrial RNA polymerases. To date, three genes for this type of RNA polymerase have been identified in the *A. thaliana* genome. Among them, RpoTm and RpoTp are exclusively imported into mitochondria and chloroplasts, respectively, while RpoTmp is imported into both mitochondria and chloroplasts (Hedtke et al., 2000).

The *rpoTp* mutant showed pale-green cotyledons and leaves, as well as severe growth retardation with a crumpled leaf phenotype, while the *rpoTmp* mutant showed only a moderately reduced growth phenotype (Baba et al., 2004; Courtois et al., 2007; Hricova et al., 2006).

Analysis of PEP-deficient mutants revealed that NEP is involved in transcription of nonphotosynthetic genes including *rpoB* (RNA polymerase), *rps15* (ribosomal protein), *accD* (fatty acid metabolism), and *clpP* (protein degradation). Type I promoters (*accD* and *rpoB*) characterized by a consensus YRTA motif are located closely upstream of transcription initiation sites of NEP-dependent genes (Silhavy and Maliga, 1998). Nonconsensus-type promoters such as *clpP* –59 (type II) and *rrn* PC promoters were also identified as specific promoters for NEP (Sriraman et al., 1998; Bligny et al., 2000). Recently, molecular genetic analyses indicated that RpoTmp is required for specific transcription from the *rrn* PC promoter while the other type I and II genes are transcribed by RpoTp (Courtois et al., 2007; Swiatecka-Hagenbruch et al. 2008).

NEP is thought to have originated from duplication of the gene for the mitochondrial RNA polymerase after endosymbiosis of the ancestral cyanobacteria. However, it is still unclear when this event occurred during evolution. In *C. merolae*, there is only one gene encoding a phage-type RNA polymerase that possibly functions at least in mitochondria, since no genes for an RNA polymerase are located on the mitochondria genome (Matsuzaki et al., 2004; Ohta et al., 1998). Whether this RNA polymerase can also transcribe chloroplast genes remains to be determined, while putative promoter structures for the T7 phage-type RNA polymerase, as well as the bacterial RNA polymerase, were predicted in the chloroplast genome of several kinds of red algae including *C. merolae* (Oudot-Le Secq et al., 2007). In contrast, PEP-deficient mutants have not been obtained in the green alga *C. reinhardtii* (Fischer et al., 1996), suggesting that PEP has an essential role and therefore NEP does not function in the chloroplast of this alga. In case of the moss *P. patens*, there are two genes for the T7 phage-type RNA polymerase; however, arguments for both subcellular localization and activity of these two gene products in chloroplasts are not yet conclusive (Kabeya and Sato, 2005; Richter et al., 2002). To understand when NEP began to be involved in transcription of chloroplast genes during the course of evolution is one of the essential subjects to consider in the evolution of the genetic system in chloroplasts.

Interestingly, a truncated form of the mitochondrial RNA polymerase in mammals was also found to be involved in transcription of nuclear genes (Kravchenko et al., 2005). This RNA polymerase, named RNAP-IV, is expressed from an alternative transcript of the gene for the mitochondrial RNA polymerase, which lacks the 262 amino acids from the amino-terminal that includes the mitochondrial targeting signal, and thus localizes in the nucleus. Although similar cases in plants have not been reported, nuclear-encoded phage-type RNA polymerase(s) might be involved in the transcription of the nuclear genome, as well as that of the mitochondrial and chloroplast genomes.

4. Plastid Transcription Factors

As mentioned above, transcription of chloroplast genes is mediated by RNA polymerases of two different origins: NEP and PEP. In addition, multiple sigma factors of PEP are involved in transcription regulation via different sets of promoters. In addition to such a complex genetic system, specific transcription factors regulating a group of chloroplast genes are also included (Fig. 1). Here, we summarize these specific transcription factors in red algae and green plants, and their functions in chloroplast transcription regulation.

4.1. TRANSCRIPTION FACTORS IN RED ALGAE

In the case of *C. merolae*, there are 5,331 genes on 16,546,747 bp of the nuclear genome (Matsuzaki et al., 2004; Nozaki et al., 2007), and only about 100 genes for transcription factors have been identified. Among them, other than four genes encoding sigma factors of the plastid RNA polymerase, no transcription factors that might function in mitochondria or chloroplasts could be found, suggesting that almost all transcription factors in *C. merolae* function to regulate transcription of the nuclear genome. However, it should be noted that this view is still speculative, because detailed functional analyses of these genes have not yet been performed. In contrast, there are four characteristic transcription factor genes (*ycf27–30*) of bacterial origin on the chloroplast genome of red algae, including *C. merolae* (Ohta et al., 2003). Since these genes are not found on chloroplast genomes of green lineage, red algal chloroplasts can be considered to be more primitive. This is also supported by several features such as gene contents of nuclear and chloroplast genomes, as described above.

Ycf27 and Ycf29 are response regulators composing the two-component regulatory system that is one of the major signal transduction systems conserved between bacteria and higher plants (Ashby and Houmard, 2006). Ycf27 and Ycf29 belong to OmpR and NarL subfamilies, respectively. Orthologous genes in the genome of the cyanobacterium, *Synechocystis* sp. PCC 6803, were found as *rpaA* (*rre31*) and *rpaB* (*rre27*) for *ycf27*, and *rre1* for *ycf29* (Ashby et al., 2002). In *Synechocystis* sp. PCC 6803, RpaA and RpaB have reported to be complementally involved in state transition of phycobilisome association (Ashby and Mullineaux, 1999); however, detailed molecular mechanisms for this regulation are still unclear. Recently, in *Synechococcus elongatus* PCC 7942 as well as in *Synechocystis* sp. PCC 6803, it has been shown that RpaB could directly bind to the promoter region of several genes dependent on high-light stress conditions (Kappell and van Waasbergen, 2007; Seki et al., 2007). Short repeated sequences, named high-light regulatory 1 (HLR1) elements (Eriksson et al., 2000; Kappell et al., 2006) are involved in this specific binding by RpaB. In the case of *C. merolae* chloroplasts, neither target genes for Ycf27 and Ycf29 nor high-light-dependent genes have been identified yet. Understanding

the significance of conservation of a two-component regulatory system even after endosymbiosis, as well as detailed molecular mechanisms of Ycf27- or Ycf29-dependent transcription, are important subjects for future research. In addition, Ycf26 (a homolog of Hik33 in *Synechocystis* sp. PCC 6803), which is encoded on the plastid genome of some red algae including *C. caldarium* (Glöckner et al., 2000), is speculated to function as a candidate of a corresponding histidine kinase for Ycf27 and/or Ycf29. In the case of *C. merolae*, however, a gene for Ycf26 was not found on the plastid genome, while a single gene encoding a putative histidine kinase, which is distinct from Ycf26, is located on the nuclear genome (Matsuzaki et al., 2004). These results suggest that the two-component regulatory system in red algal chloroplasts is composed of one or two histidine kinases and two response regulators (Ycf27 and Ycf29). Their roles in signal transduction and transcription regulation are to be further investigated.

Ycf28 is a CRP-type transcription factor, which is a homolog of cyanobacterial NtcA. Ycf30 is a LysR-type transcription factor, which is homologous to CbbR in cyanobacteria (Maier et al., 2000). They are possibly involved in transcription regulation in response to deprivation of nitrogen and carbon source. However, their functions in red algal chloroplasts remain to be elucidated.

4.2. TRANSCRIPTION FACTORS IN GREEN PLANTS

No genes encoding transcription factors are located on the chloroplast genome of green plants. In contrast, several transcription factors encoded by the nuclear genome were found to be involved in transcription regulation in chloroplasts. For example, PTF1 in *A. thaliana* is a bHLH transcription factor localizing in chloroplasts, which regulates transcription from the *psbD* light-responsive promoter (LRP) via specific binding to the AAG box on this promoter (Baba et al., 2001). The other example is CDF2 (Bligny et al., 2000), which is involved in transcription of ribosomal RNA genes by repression from PEP promoters, resulting in transcription activation from the PC promoter that is mediated by RpoTmp, as mentioned above. Other than those, several proteins that form a chloroplast nucleoid such as CND41 (Nakano et al., 1997) and PEND (Sato et al., 1998) proteins also appeared to regulate transcription in chloroplasts. Biochemical and proteomic analyses revealed that several pTAC proteins, which compose DNA–protein complexes in transcriptionally active chromosomes in chloroplasts, are thought to be involved in transcription regulation, while most of the components are not transcription factors themselves (Pfalz et al., 2006). Moreover, recent bioinformatic studies suggested that many types of nuclear-encoded transcription factors could function in chloroplasts or mitochondria (Schwacke et al., 2007; Wagner and Pfannschmidt, 2006), while further molecular characterization is required to understand their roles in chloroplast transcription regulation.

5. Nuclear Control of Plastid Transcription

As shown earlier, transcription regulation of the chloroplast genome is complicated. The components of transcription and the transcriptional system appears to differ between red algae and green plants, as does genome organization in chloroplasts. In this section, we discuss some aspects of nuclear control in transcription regulation of chloroplast genes, which is required for coordinated expression of nuclear and plastid genomes (Fig. 2).

5.1. PLASTID TRANSCRIPTIONAL REGULATION IN RED ALGAE

In the case of *C. merolae*, four sigma factor genes are encoded by the nuclear genome, suggesting that it is possible to find multiple sigma factors on the nuclear genome of other red algae as well. This fact indicates that sigma factors can function as one of the major mediators of nuclear information to regulate transcription of chloroplast genes. For example, we examined light-dependent transcription patterns in chloroplasts using DNA microarray analysis (Minoda et al., 2005). We found that transcription of almost all genes was activated in response to light, while the time course of each gene's activation pattern could be largely classified into one of the two groups: pattern I genes, of which transcripts increased at 1 h after dark-to-light

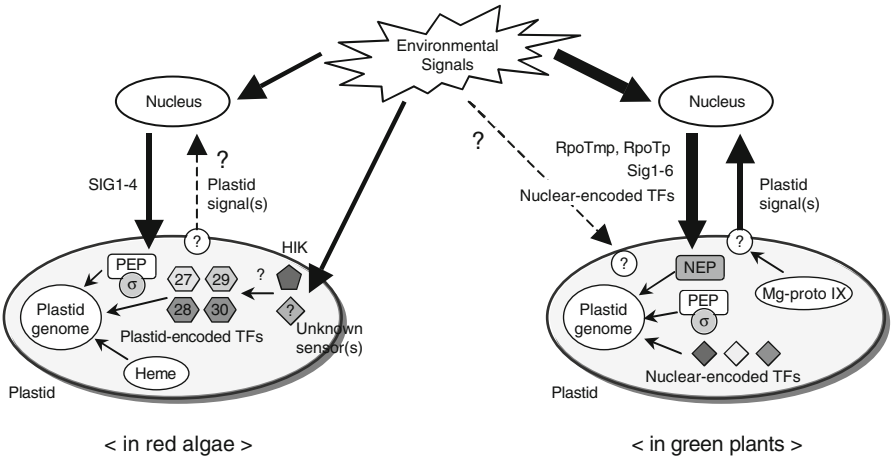

Figure 2. Model for communication between nuclear and chloroplast gene expression. In red algae (*C. merolae*, *left*), nuclear signals can be mainly mediated by sigma factors. In addition, activity of plastid-encoded transcription factors may be autonomously regulated within chloroplasts by some environmental signals. Heme is also reported to regulate a set of genes encoding phycobiliproteins. In green plants (*A. thaliana*, *right*), chloroplast transcription is more extensively controlled by the nucleus. Several types of plastid signals that regulate nuclear gene expression are also reported, including Mg-proto IX.

shift (L1), but then decreased at 6 h after dark-to-light shift (L6); and pattern II genes, of which transcripts increased at L1, then increased further at L6.

Expression of four nuclear-encoded sigma factor genes was found to be similarly activated by light. This observation suggested that light-dependent expression of chloroplast genes was mainly mediated by activation of nuclear-encoded sigma factors. Among the four sigma factors, the mRNA level of *CmSIG2* was specifically enhanced by high-light treatment, suggesting that CmSIG2 is a stress-dependent sigma factor to regulate a set of genes required for high-light tolerance in *C. merolae* chloroplasts (Minoda et al., 2005). The specific functions of the other sigma factors remain to be investigated. Interestingly, a single gene encoding a sigma factor has been found on the nucleomorph genome in the cryptophyte *Guillardia theta* (Douglas et al., 2001), suggesting that nuclear, as well as nucleomorph-encoded sigma factors, appear to regulate transcription of chloroplast genes.

In red algae, no information has been obtained as yet concerning other nuclear components for chloroplast transcription, such as NEP or specific transcription factors. On the other hand, four transcription factors (Ycf27–30) of bacterial origin are located on the chloroplast genome (Ohta et al., 2003). They may function to regulate chloroplast transcription independent of nuclear control in response to environmental changes. These characteristics suggest that the genetic system in red algal chloroplasts is more autonomous than that in green plants.

5.2. PLASTID TRANSCRIPTIONAL REGULATION IN GREEN PLANTS

In contrast to red algae, the contribution of nuclear control in transcription regulation of chloroplast genes appears to be greater in green plants. As described above, no genes for transcription regulators are found on the chloroplast genome. Transcription of chloroplast genes is mediated by two different types of RNA polymerase, NEP and PEP, and in the case of *A. thaliana*, these RNA polymerases are further divided into two types of NEP (RpoTp and RpoTmp) and six types of PEP (PEP with one of six sigma factors). Therefore, eight different kinds of RNA polymerase in total are involved in chloroplast transcription. In addition, specific transcription factors can mediate transcription from a set of promoters in response to developmental and environmental signals. These aspects indicate that transcription of chloroplast genes in green plants is more extensively controlled by the nucleus.

In higher plants like *A. thaliana*, plastids are differentiated from immature proplastids into chloroplasts, etioplasts, amyloplasts, chromoplasts, and so on, in response to environmental and developmental signals coupled with cellular differentiation. Transcription regulation during the course of such chloroplast differentiation has been well characterized. Similarly in red algae, one of the major regulatory systems of nuclear control in green plants is mediated by PEP sigma factors. Roles for multiple sigma factors are better understood in green plants than in red algae (reviewed in Kanamaru and Tanaka, 2004; Lysenko, 2007). In particular, molecular genetic analyses using *A. thaliana* or rice mutants deficient in individual sigma factor

genes revealed target plastid genes and promoters dependent on each sigma factor, such as *psaA-psaB* for Sig1 (Tozawa et al., 2007), several tRNA genes, *psaJ*, *psbD* –256 for Sig2 (Hanaoka et al., 2003; Kanamaru et al., 2001; Nagashima et al., 2004a), *psbN* for Sig3 (Zghidi et al., 2007), *ndhF* for Sig4 (Favory et al., 2005), *psbD* LRP for Sig5 (Nagashima et al., 2004b; Tsunoyama et al., 2004), and many PEP-dependent genes for Sig6 only during early chloroplast development (Ishizaki et al., 2005; Loschelder et al., 2006). Among them, Sig2 and Sig6 regulate expression of several tRNA genes including *trnE*, the gene product of which, $tRNA^{Glu}$, is required for normal chloroplast development regulating chlorophyll biosynthesis as well as chloroplast translation. Furthermore, this $tRNA^{Glu}$ was found to be a negative regulator of NEP RNA polymerase, which mediates transcription switching from NEP to PEP in the course of light-dependent chloroplast development (Hanaoka et al., 2005).

Similar to red algae, expression of all sigma factor genes is induced by light (Fujiwara et al., 2000; Kanamaru et al., 1999). Among them, expression of the Sig5 sigma factor is specifically induced by cryptochrome-mediated blue light signals as well as abiotic stresses such as high light, low temperature, or high salt conditions (Mochizuki et al., 2004; Nagashima et al., 2004b; Tsunoyama et al., 2002, 2004). These findings indicate that sigma factors are one of the important mediators of nuclear information into chloroplasts, and are thus involved in coordinated expression of both genomes in response to developmental and environmental signals.

As for NEP, both RpoTp and RpoTmp were found to be required for normal development (Baba et al., 2004; Courtois et al., 2007; Hricova et al., 2006). Specific promoters for each polymerase were recently identified by primer extension analyses (Courtois et al., 2007; Swiatecka-Hagenbruch et al., in press); however, very little information regarding general transcription regulator(s) specific for NEP has been reported. Further investigation is necessary to understand the entire picture of the chloroplast transcription system controlled by the nucleus.

6. Transcription Regulation of Nuclear Genes by Plastid-Derived Signals

As described above, transcription regulation of chloroplast genes is controlled by many kinds of nuclear components such as sigma factors of PEP RNA polymerase, which enable establishment of the coordinated expression of genes encoded on both nuclear and chloroplast genomes. Other than transcription regulation, nuclear factors also play pivotal roles in the control of various chloroplast functions. On the other hand, several studies revealed that chloroplast-derived signals might regulate expression of some nuclear genes encoding chloroplast proteins (Fig. 2). These novel types of signals are known as "plastid signals," and this regulatory system is well characterized mostly in green plants (reviewed in Nott et al., 2006; Pesaresi et al., 2007).

When chloroplast functions were inhibited by addition of chemicals such as norflurazon, an inhibitor of carotenoid biosynthesis, expression of nuclear-encoded photosynthesis genes such as *LhcB* and *RbcS* was significantly repressed even under

light conditions. This observation suggested that chloroplast conditions could be reflected in nuclear gene expression by signals of chloroplast origin. To identify candidates of these signals, a genetic approach was used to isolate mutant lines that showed constant expression of nuclear photosynthesis genes in *A. thaliana* (Susek et al., 1993). These mutant alleles were known as *genome uncoupled* (*gun*), which were deficient in five loci (*gun1–gun5*). Among them, four genes (*GUN2–GUN5*) have been found to be involved in tetrapyrrole biosynthesis, and magnesium protoporphyrin IX, one of intermediates of this pathway, has been speculated to be a possible candidate of a plastid signal (Larkin et al., 2003; Mochizuki et al., 2001). In addition, GUN1, a PPR protein localizing in chloroplasts, and ABI4, an AP2-type nuclear transcription factor, were recently found to be required for this signal transduction and nuclear transcription regulation (Koussevitzky et al., 2007).

Other than the tetrapyrrole signal, several signals derived from chloroplast translation, redox status, and oxidative stress conditions in chloroplasts are hypothesized to be other types of plastid signals (Pesaresi et al., 2007). However, the molecular characteristics for their signaling mechanisms are still unclear.

In red algae as well as in green plants, these kinds of plastid signals may function as regulators of nuclear gene expression. How, when and which types of signals act in chloroplast-to-nucleus signaling in red algae is an important question for the future. It is very interesting that a tetrapyrrole compound, which is related to the plastid signal in green plants, appears to be involved in transcription regulation in red algae also. In this case, heme in *G. sulphuraria* has been found to regulate expression of a set of genes encoding phycobiliproteins on the chloroplast genome (Troxler et al., 1989). Northern analysis demonstrated that expressions of several photosynthesis genes were similarly activated depending on light. Among them, mRNAs for allophycocyanin and phycocyanin genes were specifically accumulated in the cells incubated with heme, even in darkness. It is unknown whether this regulation also influences nuclear gene expression; however, this finding suggests that the tetrapyrrole biosynthesis pathway is strongly correlated with regulation of gene expression via cellular signaling mechanisms in both red algae and green plants.

7. Summary and Conclusion

In this chapter, we briefly focused on chloroplast genomes and their transcription regulation in red algae and green plants. Two types of RNA polymerase, multiple sigma factors, as well as specific transcription factors encoded on both nuclear and chloroplast genomes, are involved in this regulation in response to various environmental conditions and in different developmental stages. Many chloroplast functions are accomplished by proteins encoded separately on nuclear and chloroplast genomes. Mechanisms for interorganellar communication are thus very important to establish coordination of gene expression from both genomes. The regulatory mechanisms appear to differ between red algae and green plants,

which might be reflected by their growth habitats or cell morphology. Owing to advances in genomic analysis, major components for transcription regulation are largely identified, but their functions are only partly understood. Further functional evaluation of these regulatory factors, as well as studies on nuclear–chloroplast communication, are necessary to understand the complete regulatory system and the evolutionary differences between red algae and green plants.

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PLASTID EVOLUTION AND THE NUCLEAR GENOMIC "FOOTPRINT" OF RED AND GREEN ALGAL ENDOSYMBIONTS

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1. Introduction

The Russian botanist Constantin Mereschkowski was the first to speculate on the evolutionary significance of similarities between free-living cyanobacteria and the plastids (chloroplasts) of plants and algae (Mereschkowsky, 1905). The evidence that these important photosynthetic organelles evolved from prokaryotic phototrophs is now beyond refutation and a basic understanding of the process of endosymbiosis is in hand. At the genetic and cell biological level, the integration of eukaryotic host and cyanobacterial endosymbiont involves the movement of genetic material from the latter to the nuclear genome of the former, together with the evolution of a protein targeting apparatus capable of importing the protein products of essential transferred genes back to the endosymbiont-turned-organelle. Modern-day plastids retain a genome, but it pales in comparison to that from which it evolved, and hundreds of nucleus-encoded proteins must be imported from the host cytoplasm to maintain plastid function (Gould et al., 2008).

Subsequent to the evolution of plastids from cyanobacteria, plastid-bearing eukaryotes have themselves become endosymbionts inside unrelated eukaryotic hosts and over time have been converted to "secondary" or "complex" plastids (Reyes-Prieto et al., 2007). Secondary plastid-containing organisms are some of the most ecologically significant phototrophs on the planet, yet relatively little is known about their origins and diversification. This chapter focuses on the impact of secondary endosymbiosis on the host cell nuclear genome. As we shall see, the transfer of both prokaryotic and eukaryotic genes from endosymbiont to host add another layer of complexity to these already complex cells and complicates our ability to accurately infer the origin of secondary plastid-containing organisms.

2. Origin and Evolution of Plastids

The modern era of molecular biology and comparative genomics has made it possible to understand the evolutionary history of photosynthetic eukaryotes with unprecedented clarity. Building upon decade's worth of biochemical and

cell-biological investigations, we have now come to appreciate that all plastids very likely share a common endosymbiotic origin, perhaps 1.5 billion years ago (Yoon et al., 2004; Reyes-Prieto et al., 2007). Three distinct lineages diversified after this fundamental "primary" endosymbiotic event, the green algae (and their land plant descendents), the glaucophytes (or glaucocystophytes), and the subject of this book, the red algae. However, when discussing the full range of eukaryotic photosynthetic diversity, it is necessary to distinguish between two different types of plastids, those that evolved directly from cyanobacteria (i.e., primary plastids), and those that have been passed from one eukaryote to another by "secondary" or "tertiary" endosymbiosis. Both green and red algal plastids are known to have spread by this mechanism, the latter giving rise to the bulk of the diversity of eukaryotic phototrophs in the world's oceans (see later). Before focusing on the genomic aspects of secondary endosymbioses involving red algal endosymbionts, we provide an overview of the salient biochemical and cell-biological aspects of primary and secondary/tertiary endosymbiosis.

2.1. PRIMARY ENDOSYMBIOSIS

While the fine details surrounding the endosymbiotic origin of mitochondria are still debated (Embley and Martin, 2006), a relatively clear picture of the origin of plastids has emerged. Plastids are demonstrably cyanobacterial in nature and undoubtedly evolved within the confines of a fully developed eukaryote subsequent to the deepest divisions in the evolution of eukaryotic life. Instead of being digested by its heterotrophic host, the cyanobacterial progenitor of the plastid was retained, perhaps initially simply because it managed to escape the confines of the phagocytic membrane that surrounded it, and as a system for the efficient exchange of materials between the two cells evolved, the host was able to reap the full benefits of autotrophy. Today, all known primary plastids are surrounded by two membranes, which are believed to correspond to the inner and outer membranes of the cyanobacterial endosymbiont. The plastids of glaucophyte algae also possess a layer of peptidoglycan between the two membranes (Bhattacharya and Schmidt, 1997), as seen in modern-day cyanobacteria.

The transition from free-living prokaryote to organelle involved the mass transfer of genetic material from the cyanobacterial endosymbiont to the nucleus of the host. As these genes were transferred and lost from the endosymbiont genome, a protein import system evolved such that the plastid could be supplied with the essential proteins it was no longer able to produce. The plastid protein import system is composed of the Tic and Toc complexes (Translocators of the Inner and Outer Chloroplast membranes, respectively), each of which comprises multiple proteins and which mediate the import of nucleus-encoded proteins that possess amino (N)-terminal transit peptides (Bock and Timmis, 2008; Gould et al., 2008). The origin of the Tic and Toc complexes is at present poorly understood, though it appears to be a mixture of proteins derived from both the host

and endosymbiont (McFadden and van Dooren, 2004). Interestingly, not all plastid-localized proteins possess an obvious N-terminal transit peptide, suggesting the existence of alternative pathways for plastid protein targeting. In fact, recent proteomic analyses of *Arabidopsis* and rice have shown that less than 75% of putative plastid proteins possess obvious transit peptides and some appear to be targeted to the plastid via the secretory pathway (Jarvis, 2004; Kleffmann et al., 2004; Millar et al., 2006; Radhamony and Theg, 2006).

2.2. SECONDARY AND TERTIARY ENDOSYMBIOSIS

Secondary endosymbiosis occurs when a eukaryotic host cell takes up a eukaryotic photosynthetic endosymbiont, i.e., a primary plastid-containing alga, and "converts" it into an organelle (Reyes-Prieto et al., 2007; Archibald, 2009). This process is much more complex than primary endosymbiosis, as the engulfed alga brings with it a nucleus and mitochondria in addition to its plastid. The end result of most secondary endosymbioses is a plastid that is surrounded by one or more additional membranes (i.e., 3 or 4 in total); the telltale features of the engulfed eukaryotic cell, such as its endomembrane system and, in most cases, its nucleus, have all been lost. Rarely, the algal nucleus is retained in close association with the plastid in a highly reduced form known as a nucleomorph (Archibald, 2007). Nucleomorph genomes are extremely reduced relative to the genomes of their algal progenitors (see below) and in all cases examined to date are composed of three small chromosomes, ranging in size between ~100 and 200 kb (Rensing et al., 1994; Douglas et al., 2001; Gilson et al., 2006; Lane and Archibald, 2006; Lane et al., 2006; Archibald, 2007; Lane et al., 2007).

While it is generally believed that the primary endosymbiotic event that gave rise to the primary plastids of red, green, and glaucophyte algae occurred only once (Palmer, 2003), there is still much debate as to how many secondary endosymbioses have occurred during eukaryotic evolution. With respect to the evolution of green algal-derived secondary plastids, comparative genomic analyses suggest that two separate secondary events involving different green algal endosymbionts gave rise to the plastids in the chlorarachniophyte and euglenophyte lineages (Rogers et al., 2007; Takahashi et al., 2007).

In the case of red algal-derived secondary plastids, the situation is much less clear. Red secondary plastids are found in a vast array of algae, including the cryptophytes (which retain a nucleomorph), haptophytes, heterokonts (photosynthetic stramenopiles), apicomplexans, dinoflagellates, and the newly discovered genus *Chromera* (Moore et al., 2008). Two opposing views on the origin of red secondary plastids dominate the primary literature. The chromalveolate hypothesis (Cavalier-Smith, 1999) posits that they evolved only once, in a common ancestor shared by all the organisms that possess them. Alternatively, two or more separate secondary endosymbiotic events could have given rise to the plastids in each lineage. Distinguishing between these two models has proven difficult. The chromalveolate

hypothesis is considered problematic by many researchers in the field because it demands that plastids have been lost on numerous occasions. This is because some lineages such as the dinoflagellates and heterokonts are composed of both photosynthetic and nonphotosynthetic lineages, some of which appear to lack plastids entirely. A detailed discussion of the evidence for and against these competing hypotheses is beyond the scope of this chapter; interested readers are referred to any one of several recent articles on the subject (Reyes-Prieto et al., 2007; Sanchez-Puerta and Delwiche, 2008; Archibald, 2009; Bodyl et al., 2009). As will be elaborated later, the history of gene transfer from the endosymbiont to the host in such organisms has the potential to help us understand how many times red algal-derived secondary plastids have evolved during eukaryotic evolution.

To complicate matters further, several lineages of dinoflagellate algae possess plastids derived from the process of tertiary endosymbiosis in which a secondary plastid-containing alga is taken up and retained by a photosynthetic or nonphotosynthetic eukaryote. For example, the dinoflagellate *Karlodinium micrum* possesses a plastid of apparent haptophyte origin (Tengs et al., 2000), and members of the genus *Peridinium* harbor an essentially unreduced albeit permanent diatom "plastid" (Chesnick et al., 1997). The dynamic nature of plastids in dinoflagellates has been the subject of much discussion (Hackett et al., 2004), and identifying the origin of endosymbiont-derived genes in the nuclear genomes of the different dinoflagellate lineages will be an enormous challenge. Cryptic tertiary endosymbiosis has also been proposed to explain the presence of plastids in other chromalveolate lineages, including haptophytes and *Chromera* (Bodyl et al., 2009).

The extra membranes surrounding secondary and tertiary plastids significantly complicate the process of protein import in the organisms that possess them. Prior to interacting with the Tic and Toc complexes found on the inner and outer plastid membranes, host nucleus-encoded proteins must first be transported across one or two additional outer membranes. This is accomplished by taking advantage of the signal peptide secretion system of the host. Such proteins possess bipartite N-terminal leader sequences, in which the canonical transit peptide is preceded by a signal peptide, which directs the co-translational insertion of the protein into the lumen of the ER. From here, the protein may be transported to the plastid by vesicles (e.g., in chlorarachniophytes) or from the ER directly to the plastid in cases where the outermost plastid membrane is continuous with the nuclear envelope and ER (e.g., as in cryptophytes, heterokonts, and haptophytes) (see (McFadden, 1999; Gould et al., 2006, 2008) and references therein for comprehensive review).

3. The Genomic "Footprint" of Endosymbiosis: How Big and How Important?

Part-and-parcel with the evolution of an endosymbiotically derived organelle is the intracellular movement of DNA from endosymbiont to host. This process is referred to as endosymbiotic gene transfer (EGT, Martin et al., 1993; Timmis et al., 2004;

Kleine et al., 2009) and, in the case of plastids, has reduced the coding capacity of modern-day organelles to at most ~200 proteins (Martin et al., 1998, 2002). Given that well over 1,000 proteins are required to maintain plastid function, most plastid proteins are nucleus-encoded, synthesized on cytosolic ribosomes, and targeted to the organelle post-translationally. Beyond serving as a repository for hundreds of genes for essential plastid proteins, what is known about the impact of EGT on the nuclear genome of primary and secondary plastid-containing organisms?

3.1. CYANOBACTERIAL CONTRIBUTIONS TO THE NUCLEAR GENOME

Several studies have attempted to quantify the contribution of the cyanobacterial progenitor of the plastid to the nuclear genomes of plants and algae. Building on previous data showing a mosaic evolutionary history of nuclear genes for both cytosolic- and plastid-localized metabolic enzymes in plants (Martin et al., 1996; Martin and Schnarrenberger, 1997), Martin et al. (2002) performed a comprehensive analysis of the *Arabidopsis* nuclear genome and inferred that ~18% of its genes – ~4,500 of ~25,000 in total – appear to be derived from cyanobacteria. This is a remarkably high number, larger in fact than the total gene repertoire of many present-day cyanobacterial genomes. Unexpectedly, fewer than 50% of the "cyanobacterial" genes in *Arabidopsis* were found to encode proteins with obvious plastid-targeting signals, with the majority instead being implicated in a wide variety of host-associated processes including intracellular transport and cell division (Martin et al., 2002).

A more recent and comprehensive analysis of *Arabidopsis*, rice, the green alga *Chlamydomonas*, and the red alga *Cyanidioschyzon* has produced a slightly lower estimate, with ~14% of the genes in the nuclear genomes of these species touted as being cyanobacterial in origin (Deusch et al., 2008). An analysis of an expressed sequence tag (EST) data set from the glaucophyte alga *Cyanophora* came up with an estimate of 11% cyanobacterial genes and in contrast to the prediction for *Arabidopsis*, less than 10% of these genes were predicted to encode plastid proteins. The reason(s) for this discrepancy is open to speculation, but regardless, the cyanobacterial genomic footprint in plant and algal genomes is impressive and apparently unrestricted with respect to biological function. The contribution of the cyanobacterial endosymbiont to the biochemistry and cell biology of its host would appear to extend far beyond photosynthesis and plastid-localized processes (Martin et al., 2002; Archibald, 2006).

3.2. EUKARYOTE–EUKARYOTE GENE TRANSFERS AND REPLACEMENTS

By definition, secondary endosymbionts possess a nucleus and mitochondria in addition to a plastid. Consequently, the impact of secondary endosymbiosis on the host nuclear genome is conceivably orders of magnitude greater than in the

case of primary endosymbiosis. Given that in most secondary plastid-containing algae, the endosymbiont nucleus has been eliminated, and that most of the essential cyanobacterial genes for plastid proteins would already have been nucleus-encoded prior to secondary endosymbiosis, extensive nucleus-to-nucleus gene transfer must go hand-in-hand with secondary endosymbiont integration. The complete genome sequences of red secondary plastid-containing organisms such as the heterokont *Thalassiosira* (Armbrust et al., 2004) and the apicomplexan *Plasmodium* (Gardner et al., 2002) show this to be true.

Even in the cryptophyte and chlorarachniophyte algae where the secondary endosymbiont nucleus persists, nucleomorph genomes sequenced to date are extremely gene poor, encoding at most ~600 proteins and 30 plastid-targeted proteins (Douglas et al., 2001; Gilson et al., 2006; Lane et al., 2007). Nevertheless, by virtue of the fact that both lineages retain a nucleomorph and residual endosymbiont cytoplasm surrounding the plastid (the periplastid space), cryptophytes and chlorarchniophytes are unlike all other secondary plastid-containing algae in that core processes such as DNA replication, transcription, and translation occur simultaneously in two distinct nucleocytoplasmic compartments. Endosymbiont-derived, host-nucleus-encoded gene products of *eukaryotic origin* must therefore be targeted to the organelle (Archibald, 2007).

Conversely, there is at present little in the way of information on the extent to which the nuclear genomes of red algal endosymbionts (in "chromalveolate" taxa) and green algal endosymbionts (in the chlorarachniophytes and euglenids) have contributed to the biology of their hosts, in the same way as the cyanobacterial progenitor of plastids appears to have done for primary plastid-containing organisms (Martin et al., 2002; Deusch et al., 2008). It is safe to assume that during the process of secondary endosymbiosis, the host nucleus is bombarded with the entire contents of the algal endosymbiont's nuclear genome and it seems likely that at least some of these transferred genes would acquire a novel function or take over the function of a host nuclear counterpart. Such "endosymbiotic gene replacements" (EGRs) are known to have occurred in the case of cyanobacterial-derived genes (Martin and Schnarrenberger, 1997) and should be even easier in the context of secondary endosymbiosis, given that it involves the replacement of a eukaryotic gene/protein with a homolog from another eukaryote (Archibald and Keeling, 2003; Archibald, 2005). EGR has also been shown to work in reverse, i.e., with a host-derived gene acquiring the ability to encode a protein with the appropriate targeting information to direct it to the organelle (e.g., Fast et al., 2001; Nowitzki et al., 2004). In the case of cryptophytes and chlorarachniophytes, it is thus possible that the protein products of at least some host nucleus-derived genes are targeted to the periplastid space and nucleomorph, an idea that remains to be rigorously tested in lieu of a complete nuclear genome sequence from a nucleomorph-containing alga.

To our knowledge, no cases of EGR involving eukaryotic genes have been thoroughly investigated, although EGT is increasingly well documented. One of the first examples was the demonstration that the nuclear genomes of at least some cryptophyte algae encode two evolutionarily distinct actin isoforms, one of

which appears to be red algal in origin and thus secondary endosymbiont-derived (Stibitz et al., 2000; Tanifuji et al., 2006). The function(s) of these red algal actins in the cell biology of the cryptophyte host and/or endosymbiont are currently unknown. EST and whole genome sequence analyses have uncovered many more apparent instances of eukaryote–eukaryote EGT in the haptophyte *Emiliania huxleyi* (Li et al., 2006), the dinoflagellate *Karenia brevis* (Nosenko et al., 2006), the heterokont *Thalassiosira pseudonana* (Armbrust et al., 2004), and the green secondary plastid-containing alga *Euglena gracilis* (Ahmadinejad et al., 2007). At this stage, however, the lack of a clear picture of the extent and significance of EGTs/EGRs involving eukaryotic donor and recipient genomes is due in large part to the problem of detection. Unlike cyanobacterial genes, which stand out against a nuclear genomic backdrop, eukaryotic genes that have undergone EGT are by definition much more closely related to the genome in which they currently reside and are thus more difficult to detect as anomalous by BLAST or phylogenetic analysis. Combined with issues of taxon sampling and uncertainties surrounding the phylogenetic origins of the secondary host eukaryotes, this means that EGT and EGR are at present unknown quantities. Nevertheless, it seems likely that examples such as the cryptophyte red algal actin described above represent the tip of a large iceberg (Lane and Archibald, 2008).

4. Deep Algal Phylogenomics: Problems and Promise in Light of EGT and EGR

An exciting application of the rapidly developing discipline of "phylogenomics" (Eisen and Fraser, 2003; Delsuc et al., 2005) is to infer organismal relationships from large data sets comprising many genes concatenated together and analyzed as a single unit. Phylogenomic analyses have begun to shed light on the root structure of the eukaryotic tree of life, and contributed to the (as yet unproven) notion that eukaryotes can be split into six "supergroups," four of which contain at least one photosynthetic lineage (Simpson and Roger, 2004; Adl et al., 2005; Lane and Archibald, 2008). However, an oft-overlooked (or ignored) assumption in such analyses is that each of the genes assembled into a single supermatrix share the same evolutionary history. Given the discussion in the previous section, is this a valid assumption in the case of secondary plastid-containing algae? If the footprint of red and green algal secondary endosymbionts on the nuclear genomes of their hosts really is significant, it behooves us to consider the practical and theoretical implications of this observation for algal genomics research.

An important case in point involves the "chromalveolates," an as-yet still hypothetical assemblage of protist lineages united by the fact that (with the exception of some dinoflagellates) plastid-bearing members possess organelles derived from the uptake of a red alga (Cavalier-Smith, 1999; Reyes-Prieto et al., 2007). As mentioned earlier, the chromalveolates constitute a huge fraction of the known diversity of algae (Keeling et al., 2005), and whether these red secondary plastids evolved once or multiple times has been extensively debated in recent years

(e.g., Lane and Archibald, 2008; Sanchez-Puerta and Delwiche, 2008; Archibald, 2009; Bodyl et al., 2009; Keeling, 2009). The controversy centers mainly on the fact that many "chromalveolate" lineages are not photosynthetic and do not even possess plastids (e.g., the ciliates). Phylogenomics has been used to assess the degree of congruence between the chromalveolate host phylogeny inferred from nuclear genes and (when present) plastid loci. The goal is to determine whether a single plastid origin, combined with multiple plastid losses, is a reasonable explanation for the observed distribution of plastids and photosynthesis in chromalveolates, or whether a model of multiple independent secondary and/or tertiary endosymbioses is a better fit to the observed data.

The issue is still far from settled. While specific subsets of chromalveolate taxa, such as the cryptophytes and haptophytes, appear strongly united in phylogenomic analyses of 100+ nuclear genes (e.g. Patron et al., 2007 Burki et al., 2008), other relationships predicted to exist based on the original chromalveolate hypothesis (Cavalier-Smith, 1999), such as a tripartite assemblage of the "chromists" (i.e., cryptophytes, haptophytes, and heterokonts), are not recovered. In fact, the supergroup Rhizaria, to which the green algal plastid-containing chlorarachniophytes belong, appear more closely related in host nuclear gene phylogenies to stramenopiles and alveolates (i.e., dinoflagellates, apicomplexans, and ciliates) to the exclusion of cryptophytes and haptophytes (Burki et al., 2007, 2008). Interestingly, the cryptophyte+haptophyte clade has shown a tendency to branch with the primary-plastid-containing lineages in such analyses. One interpretation of this latter observation is that as-yet undetected EGTs/EGRs involving a subset of the genes used in the analyses could be influencing the branching positions of the chromalveolate taxa relative to one another and to red, green, and glaucophyte algae (Archibald, 2009).

Accurately determining the evolutionary position of secondary plastid-containing algae on the eukaryotic tree of life represents a huge challenge for the coming years, and will no doubt raise philosophical questions about the nature of deep eukaryotic evolution and the extent to which genome mosaicism has played a role in the evolution of both photosynthetic and nonphotosynthetic eukaryotes. Identifying instances of EGT and EGR will depend greatly on continued methodological advances as well as on the acquisition of genomic data from an even broader array of protist lineages. These include "host" groups such as poorly studied chromalveolate taxa (e.g., cryptophytes and nonphotosynthetic stramenopiles) as well as a wide range of red algae, such that the origin(s) and subsequent diversification of red algal secondary plastids can be elucidated.

Finally, while EGT/EGR certainly represents a challenge for deep eukaryotic phylogenomics, it also has the potential to enhance our ability to discern evolutionary relationships, rather than obscure them. Once endosymbiont-derived genes are identified, they have the potential to serve as discrete characters whose presence/absence in the nuclear genomes of diverse chromalveolate taxa can help us test hypotheses generated using phylogenomics. EGRs involving nuclear genes for plastid proteins have already proven useful in providing evidence in favor of the

chromalveolate hypothesis (e.g., Fast et al., 2001; Patron et al., 2004), and systematic surveys aimed at identifying EGTs and EGRs of nonplastid-associated genes in the nuclear genomes of the cryptophyte *Guillardia theta* and other "chromalveolate" taxa are currently underway. Ultimately, a comprehensive understanding of the complex ways in which host- and endosymbiont-derived genes and proteins have integrated with one another in the process of secondary endosymbiosis will provide a richer appreciation of endosymbiosis as a driving force in the evolution of life.

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RED MICROALGAE: FROM BASIC KNOW-HOW TO BIOTECHNOLOGY

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1. Preface: The Interchange Between Genes and Products

The group of Arad has taken up the challenge of exploiting the potential of the sulfated polysaccharides of red microalgae for biotechnological applications. To this end, they are conducting an integrated investigation, which initially involved chemical, physiological, and biochemical studies of the polysaccharide and the development of large-scale production technologies. However, as the group became more and more familiar with the subject, it became clear that the sulfated polysaccharides that characterize the red microalgae comprise a unique and very complex group of molecules and that biotechnology development would be correspondingly complex.

In the past few years, the group has started the complementary part of biotechnology R&D, i.e., molecular genetic research, approaching it in two ways – study of the genome and cellular manipulations. In this respect, they are only just beginning to unravel the potential of manipulating red algae for biotechnological applications.

The above difficulties notwithstanding, the main aim of biotechnology development – bringing the product to the market – has been partially fulfilled for the red microalgae. Some of the sulfated polysaccharides are already in use in various cosmetic products, and other applications are in advanced stages of development. However, the group is now at the beginning of the long road to connecting products to genes.

2. Biotechnology of Production of Valuable Chemicals from Red Microalgae

During the past few years, the Arad laboratory has been engaged in the production of bioactive materials from red microalgae. These unicells produce a variety of chemicals with biotechnological potential, particularly sulfated polysaccharides (Arad, 1999; Arad and Richmond, 2004; Arad et al., 1985; Pulz and Gross, 2004;

Ramus and Groves, 1972, 1974). It is these unique sulfated polysaccharides that have become the focus of the R&D program of the Arad laboratory. This ongoing project is a multidisciplinary effort that requires the coordination of various disciplines: chemistry (characterization of the polysaccharide), physiology (study of the algae under various growth conditions), biochemistry (unraveling the polysaccharide and sulfation pathways), biotechnology (development of applications of the various bioactivities), and engineering (development of a large-scale cultivation system). The molecular genetic studies described in the following sections have only recently become a significant part of the program.

Chemical characterization of the sulfated polysaccharide of the red microalga *Porphyridium* sp. revealed that it is an anionic heteropolymer (molecular mass 3–5 $\times 10^6$ Da) composed of about ten different sugars (Arad, 1988; Geresh and Arad, 1991; Heaney-Kieras and Chapman, 1976; Heaney-Kieras et al., 1977). The main sugars are xylose, glucose, and galactose (Arad, 1999; Geresh and Arad, 1991; Geresh et al., 1992), with the sulfate group being located on the glucose and galactose moeities (Lupescu et al., 1991). A 66-kDa glycoprotein is noncovalently bound to the polysaccharide (Shrestha et al., 2004). The polysaccharide hydrogel is negatively charged due to the presence of glucuronic acid and sulfate.

The exact structure of the red microalgal polysaccharides – their sugar sequence and three-dimensional configuration – has not been fully elucidated, but a basic disaccharide building block, 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactopyranose, which is an aldobiuronic acid, has been isolated from three different species (Geresh et al., 1990; Jaseja et al., 1989; Lupescu et al., 1992).

Based on extensive rheological studies – physicochemical characterization of polysaccharide solutions – the polysaccharide of *Porphyridium* sp. was found to have a double or triple helix configuration and to exhibit pseudoplastic, thixotropic, non-Newtonian behavior (Eteshola et al., 1996, 1998). Recently, it was shown that this polysaccharide is superior to hyaluronic acid as a lubricant (including as a joint lubricant) in terms of friction reduction, adsorption, and stability (Arad et al., 2006, Gourdon et al., 2008). One of the most important qualities of the polysaccharide is its resistance to extreme conditions (temperatures of 20–150°C, pH values of 3–8, and salinity) and to enzymatic degradation (by hyaluronidase and carbohydrolases) (Arad et al., 1993b, 2006).

Physiological studies were conducted aiming at understanding the effect of environmental conditions on quality and quantity of the polysaccharide, e.g., the effect of light (Friedman et al., 1991), N, SO4, or CO2 depletion – or enrichment – of the medium (Adda et al., 1986; Arad et al., 1988, 1992; Dubinsky et al., 1992; Li et al., 2000), continuous vs. batch mode of production (Adda et al., 1986), and mode of harvesting. These studies were part of the efforts to establish the conditions that would facilitate control of the repeatability of polysaccharide production under outdoor conditions.

For advanced biotechnological development, it is necessary to understand the biochemical pathways involved in cell wall formation. Since very little was known about the formation of red algal cell wall, the Arad laboratory took a variety of different approaches to this part of the study – investigating the involvement

of the golgi apparatus in cell wall formation and the effect of golgi inhibition by brefeldin A (BFA) (Keidan et al., 2009; Noguchi et al., 1998), following cell wall formation during the cell cycle (Simon-Bercovitch et al., 1999), and using the cell wall inhibitor 2,6-dichlorobenzonitrile (DCB) to isolate cell-wall-modified mutants (Arad et al., 1993a, 1994) as a tool for understanding cell wall biosynthesis. Elucidation of intracellular carbon partitioning towards cell wall formation revealed the importance of floridoside as a dynamic carbon pool for cell wall formation (Arad and Richmond, 2004; Li et al., 2001). Perhaps, the most important part of the biochemical pathways in the red microalgae is sulfation, a process that is unique to the red and brown algae and to mammals (Gao et al., 2000). The biochemical and genetic aspects of the biosynthesis involved in sulfation of the polysaccharides through the sulfotransferase gene (Keidan et al., 2006, Plesser et al., 2007) are currently under study in the Arad laboratory.

Because of the infancy of the subject during the initial stages of the research, any biotechnological planning required the development of large-scale cultivation system. Although various systems for commercial cultivation of microalgae have been under development for some years, accumulated experience in outdoor production of sulfated polysaccharides using vertical polyethylene sleeves – the technology developed by the Arad laboratory – was found to be the method of choice for the culture of red microalgae, and particularly for the production of the extracellular polysaccharides (Cohen and Arad, 1989; Arad and Richmond, 2004). The advantages of the sleeves over other technologies are: better light availability and improved turbulence, which affect the cells, light/dark cycles (Merchuk et al., 1998), smaller temperature fluctuations, prevention of contamination, and ease of disposability. Many aspects of large-scale production are dictated by the physiological requirements of cell growth for optimal production of a particular biochemical; for example, for the red microalgae, the time of harvesting and/or mode of harvesting (batch vs. continuous) for the production of extracellular polysaccharides, which tend to dissolve in the medium, differ from the same parameters for the production of products that accumulate in the cells, e.g. pigments. In addition to the vertical sleeve technology, the Arad group also developed a downstream filtration process for isolation, purification, and drying (Ginzberg et al., 2008) of the polysaccharides.

It became clear early in the research that red microalgal sulfated polysaccharides are novel molecules with special bioactivities. They were found to have anti-inflammatory and soothing activity (Matsui et al., 2003) and antioxidant properties (Tannin-Spitz et al., 2005), which made them suitable for use in dermal applications. Indeed, the sulfated polysaccharide of *Porphyridium* sp. is already in use in more than 100 products of a world-renowned cosmetic company. This polysaccharide was also found to have antiviral activity against *Herpes simplex* viruses (types 1 and 2) and *Varicella zoster* (Arad et al., 2006; Huleihel et al., 2001, 2002). It is believed that the sulfate is the bioactive group.

The red microalgae are also a source of other biochemicals, such as phycobiliproteins, which find application as natural colors (Yaron and Arad, 1993). The algal biomass contains a unique combination of functional sulfated polysaccharides (acting as dietary fibers), polyunsaturated fatty acids (PUFAs) (Yaron et al., 1995),

zeaxanthin, minerals, and proteins (Arad, 1988; Arad and Richmond, 2004). Experiments in which the algal biomass was fed to rodents showed a significant improvement in cholesterol metabolism – a reduction in total serum cholesterol, triglycerides, and hepatic cholesterol levels and an increase in HDL/LDL ratio, fecal excretion of neutral sterols and bile acids (Dvir et al., 1996, 2000; Ginzberg et al., 2000).

As was said earlier, developing biotechnology also requires molecular-genetic R&D. The Arad laboratory has chosen to concentrate on unraveling the red microalgal genome and developing red microalgae as cell factories. Developing the methods and technologies required for this part of the R&D is not obvious but is crucial. All these are part of this multidisciplinary development.

3. Comparative Genomics

3.1. EXPRESSED SEQUENCE TAGS (ESTS) OF RED MICROALGAE

Although a great deal of information has been accumulated on microalgal biochemistry, physiology, cell biology, and systematics, the progress in the study of the molecular genetics of microalgae (with exception of the model alga *Chlamydomonas reinhardtii*) lags far behind than that for higher plants and animals, mainly because of the lack of appropriate tools for genetic research. Recently, however, important progress has been made in the field of microalgal genomics.

To date, sequencing has been completed for only one species of red microalga – *Cyanidioschyzon merolae* (subphylum *Cyanidiophytina*) all three genome compartments – nuclear (Matsuzaki et al., 2004; Nozaki et al., 2007), mitochondrial, and plastid (Ohta et al., 2003) – have been completely sequenced. The chloroplast of the cyanidiophyte *Cyanidium caldarium* was the first red microalgal organelle genome to be sequenced (Glockner et al., 2000), and the nuclear genome sequencing of another cyanidiophyte *Galdieria sulphuraria* is currently underway (Barbier et al., 2005; Oesterhelt et al., 2008; Weber et al., 2004). For species of the subphylum Rhodophytina (Yoon et al., 2006), the chloroplast (Reith and Munholland, 1995) and the mitochondrial genome (Burger et al., 1999) of *Porphyra purpurea* (Bangiophyceae) have been completely sequenced, and there is currently a proposal on the drawing board to sequence the genome of *P. purpurea* [under the auspices of the US DOE Joint Genome Institute (JGI)].

Other algae for which near-full nuclear genome information has become – or will shortly become – available are the green alga species, *C. reinhardtii* (Merchant et al., 2007) and *Volvox carteri,* the diatoms, *Thalassiosira pseudonana* (Armbrust et al., 2004), *Phaeodactylum tricornutum, Fragilariopsis cylindrus,* and *Pseudo-nitzschia*, and the marine picoeukaryote, *Ostreococcus tauri* (Grossman, 2005, 2007).

ESTs constitute the primary tool for genomic exploration and for functional genomic projects in the absence of genome sequence data, which are unlikely to be available in the foreseeable future other than for a few model algae

(Quackenbush et al., 2001). Moreover, ESTs serve as an invaluable resource for protein identification and characterization in proteomics (Lisacek et al., 2001). In addition to functional genomics, EST projects generate an excellent means to study the evolution of genes and proteins – from unicellular photosynthetic algae towards the multicellular plants.

There are a small number of reports on EST projects of Rhodophyta: 200 ESTs of *Gracilaria gracilis* (Florideophyceae) (Lluisma and Ragan, 1997) and 20,779 ESTs of *P. purpurea* (Asamizu et al., 2003; Nikaido et al., 2000) are available. EST databases for the red microalga species *Porphyridium* sp. and *Dixoniella grisea* (formerly classified as *Rhodella reticulata*) were recently established – by the group of Arad – to complement biochemical and physiological studies of polysaccharide biogenesis with a molecular genetics approach (Fig. 1). In fact, the databases have already proven useful in the cloning of a cDNA encoding the cell-wall polysaccharide-associated glycoprotein from *Porphyridium* sp., which was predicted to be involved in polysaccharide biosynthesis (Shrestha et al., 2004).

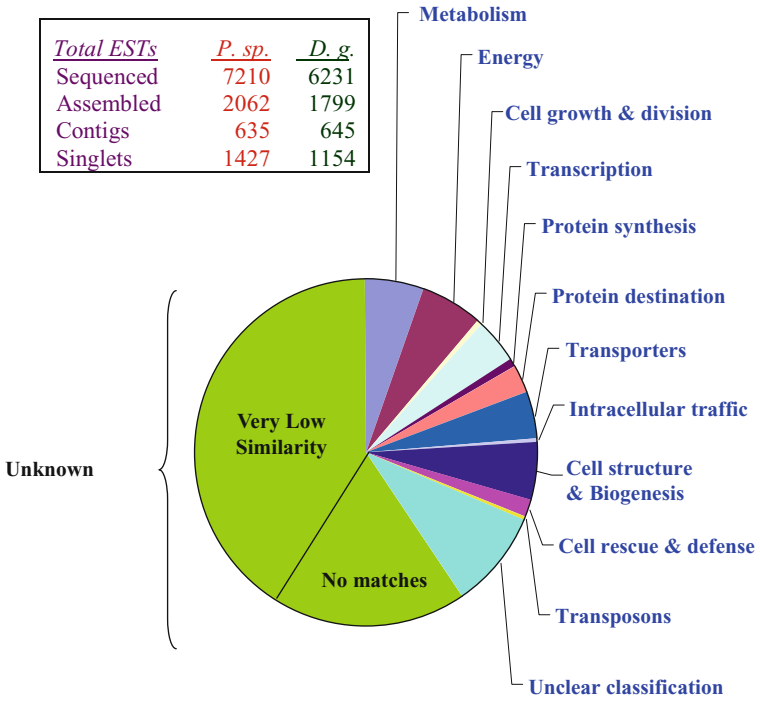

Figure 1. Functional categories of ESTs of *Porphyridium* sp. *Inset*: overview of *Porphyridium* sp. (*P.* sp.) and *Dixoniella grisea* (*D.g.*) EST sequencing projects. G/C:T/A ratio = 50:50*. *Data was calculated from 30 high-quality EST sequences (average 700 bp long).

Non-normalized unidirectional cDNA libraries constructed from algae grown under various physiological conditions generated 7,210 and 6,231 ESTs from *Porphyridium* sp. and *D. grisea*, respectively. The ESTs were assembled with the CAP3 Sequence Assembly Program (Huang and Madan, 1999), which gave 2,062 and 1,799 nonredundant sequences (unigenes) for *Porphyridium* sp. and *D. grisea*, respectively. The *Porphyridium* sp. sequences contained 635 contigs (30.8%) and 1,427 singlets (69.20%), whereas those of *D. grisea* contained 645 contigs (35.85%) and 1,154 singlets (64.15%).

Since *Porphyridium* sp. unigenes show good similarity to those of *C. merolae* (see following section) among sequenced genomes, the group of Arad used the functional categorization of the latter to assign functions to the *Porphyridium* sp. unigenes. The *C. merolae* protein database was searched using BLASTX with an E-value cutoff of 1E-07, and thus *Porphyridium* sp. unigenes were categorized according to the eukaryotic clusters of orthologous groups of proteins (KOGs) system (Fig. 1). In addition, for unigenes for which there was no sequence similarity to those of *C. merolae*, Clusters of Orthologous Groups of proteins (COGs) databases were searched by using the reverse position-specific (RPS)-BLAST algorithm (Marchler-Bauer et al., 2003). In addition, for *Porphyridium* sp. unigenes that exhibited similarity to known proteins but that were not functionally categorized in the above-mentioned databases, functions were assigned manually from information in the UniProt/TrEMBL database. Thus, of the 2,069 unigenes, 41% were assigned to known proteins. A little more than half of the *Porphyridium* sp. unigenes (59%) did not exhibit any similarity to the known proteins in the database.

3.2. NEW INFORMATION ON THE PHYSIOLOGY OF RED MICROALGAE DERIVED FROM EST DATA

While the ESTs sequenced from normalized cDNA libraries may be used to identify transcripts that are expressed in one library but absent from another, accurate quantitative data describing the relative abundance of genes within a library can be obtained only from ESTs generated from non-normalized cDNA (Moody, 2001). The strategy of the Arad laboratory to use non-normalized cDNA libraries – rather than normalized libraries – provided information on gene expression profiles under various physiological conditions.

3.2.1. Some Interesting Genes Derived from EST Databases

ADP-Ribosylation-Factor- and ADP-Ribosylation-Factor-Like Proteins

The group of Arad has prepared cDNA libraries from the logarithmic and stationary phases of *Porphyridium* sp. to identify genes expressed in specific phases of growth. While ESTs encoding ADP-ribosylation factor-like protein 1 (Arl1) and ADP-ribosylation factor-1 (Arf1) were found to be the first and third most abundant ESTs (184, 5.86% and 90, 2.87%), respectively, in the stationary phase,

Table 1. Abundance of ESTs in the logarithmic and stationary phases.

		Stationary phase		Logarithmic phase	
Contigs	**Annotation**	**Position[a]**	**%[b]**	**Position[a]**	**%[b]**
Contig 234	ADP-ribosylation factor	1	5.86	5	4.05
Contig 216	Flavohemoprotein	2	3.6	4	4.12
Contig 348	ADP-ribosylation factor	3	2.87	14	1.18
Contig 72	Cofilin	5	2.55	2	5.27
Contig 191	Flavohemoglobin Hmp	8	2.42	8	1.76
Contig 316	Blue light receptor	10	1.85	–	0

[a]Level of EST abundance.
[b]% of total ESTs from corresponding phase.

Arl1 and Arf1 occupied the 5th and 14th positions, respectively, in the logarithmic phase (Table 1).

Arf GTP-binding proteins, members of the Ras superfamily of GTPases, have well-established functions in membrane-trafficking pathways. The six Arf proteins reported from various organisms have been classified into three classes. Only one Arf1 protein – that belonging to class I – was found in *Porphyridium* sp. Among the Arl proteins, Arl1 is most similar to Arf1 (Otero and Vincenzini 2004). *Porphyridium* sp. *Arl1* and *Arf1* have 70% identity at the nucleotide level and 60% identity and 75% similarity at the amino acid level. Arl1 is involved in endosome-to-Golgi trafficking, trans-Golgi network (TGN) protein sorting and ion homeostasis (reviewed in Burd et al., 2004). In *Porphyridium,* the extracellular polysaccharide is synthesized in the Golgi apparatus and transported to cell surface via vesicles (Ramus, 1972; Ramus and Robins, 1975). The high abundance of Arl1 and Arf1 in both phases of algal culture grown at constant light, and hence with continuous carbon-fixation, indicates their role in polysaccharide biogenesis/secretion. This notion is in agreement with Arad and Richmond (2004) and Singh et al. (2000) that higher amounts of polysaccharide are produced in the stationary phase than in the logarithmic phase, and thus abundant *Arl1* and *Arf1* are required to transport the higher amounts of fixed carbon.

3.2.1.1. Flavohemoprotein/Flavohemoglobin

Hemoglobin genes have been discovered in all six kingdoms of life. The presence of hemoglobins in unicellular organisms suggests that the gene for hemoglobin is very ancient and that hemoglobins must have functions other than oxygen transport, since oxygen delivery in unicellular organisms is a diffusion-controlled process (Mukai et al., 2001). Nonvertebrate flavohemoglobins are implicated in nitric oxide detoxification in an aerobic process known as the nitric oxide dioxygenase reaction, which protects the host from various noxious nitrogen compounds (Frey and Kallio, 2003; Mukai et al., 2001), and hence play a central role in the inducible response to nitrosative stress. In *Porphyridium* sp., two flavohemoglobins

occupied the top eight positions (4th and 8th in logarithmic phase; 2nd and 8th in stationary phase) among the most abundant ESTs (Table 1). The sequences of the two flavohemoglobins have no similarity at the nucleotide level, but have 31% identity and 51% similarity at the protein level. None of them exhibited similarity to that of truncated hemoglobin. It is most likely that during intense photosynthesis, and hence during polysaccharide synthesis in *Porphyridium* sp., under nitrogen-depleted and photo-oxidative conditions, large amounts of nitric oxide free radical are produced. Abundant flavohemoglobins are therefore required for detoxification of the nitric oxide, which is a potent photosynthetic inhibitor that acts by reducing CO_2 uptake. This notion is yet to be verified experimentally. Flavohemoglobins have been found in *D. grisea* but not in other algae, including *C. merolae* and *T. pseudonana*, the two algae for which complete genome sequences are available. Furthermore, flavohemoglobins have been found only in bacteria and fungi, but not in other organisms, including plants and animals, not even archaea and cyanobacteria.

3.2.1.2. Blue Light Receptor

There are two distinct classes of photoreceptors that mediate the effects of UV-A/blue light (320–500 nm): the cryptochromes and the phototropins. While cryptochromes play a major role in plant photomorphogenesis, the phototropins are involved in regulating light-dependent processes that serve to optimize the photosynthetic efficiency of plants (reviewed in Christie, 2007). In *Porphyridium* sp., contig 316, which was the 14th most abundant EST in the stationary phase but absent in the log phase, exhibited similarity to blue light receptors. In the stationary phase, algal cells receive less light, because of the high density of the culture medium (higher cell numbers and progressively increasing viscosity of the medium). It is very likely that only blue and UV light can penetrate the viscous medium. The algal cells thus may express the blue light photoreceptor proteins to maximize the capture of the only light available for photosynthesis.

3.3. COMPARATIVE GENOMICS: RED ALGAE ARE NOT PRIMITIVE EUKARYOTES BUT SISTER GROUPS TO VIRIDAEPLANTAE

3.3.1. Similarity Between Porphyridium sp. and Dixoniella grisea

In a comparison of sequences, 2,062 unigenes of *Porphyridium* sp. were compared with 1,799 unigenes of *D. grisea*. Since the two species of microalga have not been completely sequenced, there is high probability that a number of orthologous genes from the two species have not yet been sequenced. At present, there are large numbers of unigenes of *Porphyridium* sp. and *D. grisea* for which there are no similarities (<1E-06). The two unigenes with the highest similarity were actin (e-value 0) and eukaryotic translation elongation factor 1 alpha (1e-172). Other unigenes that exhibited high similarity (1E-49–1E-50) were ribosomal proteins, histones, eukaryotic translation initiation factor eIF-6, a heat shock protein of the Hsp90 family, ubiquitin-conjugating enzyme, a small GTP-binding protein of

the Rab family, manganese superoxide dismutase, enolase, cyclophilin B, and light harvesting proteins.

3.3.2. Similarity of Porphyridium sp. and Dixoniella grisea unigenes to Various Proteomes

The Arad group compared *Porphyridium* sp. and *D. grisea* ESTs to the genome derived-proteomes of three species of microalga (*C. merolae*, *C. reinhardtii*, and *T. pseudonana*), two higher plant species (*Arabidopsis thaliana* and *Oryza sativa*), an yeast (*Saccharomyces cerevisiae*), and a nematode (*Caenorhabditis elegans*), for which nearly complete genome sequences are available. In addition, EST-derived protein sequences of *Porphyra yezoensis* and protein sequences of cyanobacteria and archaea available in Uniprot/TrEMBL databases were also compared with ESTs of *Porphyridium* sp. and *D. grisea*. Although the Cyanidiales, including *C. merolae*, are evolutionarily distantly related to the Porphyridiales (Oliveira and Bhattacharya, 2000; Yoon et al., 2002, 2006), unigenes of both *Porphyridium* sp. and *D. grisea* exhibited the best similarity to *C. merolae*, followed by the green plant *A. thaliana* (Fig. 2) and thereafter by two other species of unicellular alga, the green alga *C. reinhardtii* and the diatom *T. pseudonana.* These findings are in keeping with the idea that red algae are sister groups to green plants. The lesser similarity to *P. yezoensis* is perhaps due to the fact that this alga has not yet been completely sequenced – only 20,779 ESTs (4,602 unigenes after CAP3 assembly) are available. In contradiction to the previously held hypothesis that red algae constitute an evolutionary bridge between prokaryotes and eukaryotes, *Porphyridium* sp. and *D. grisea* were found to be less similar to prokaryotes, cyanobacteria, and archaea

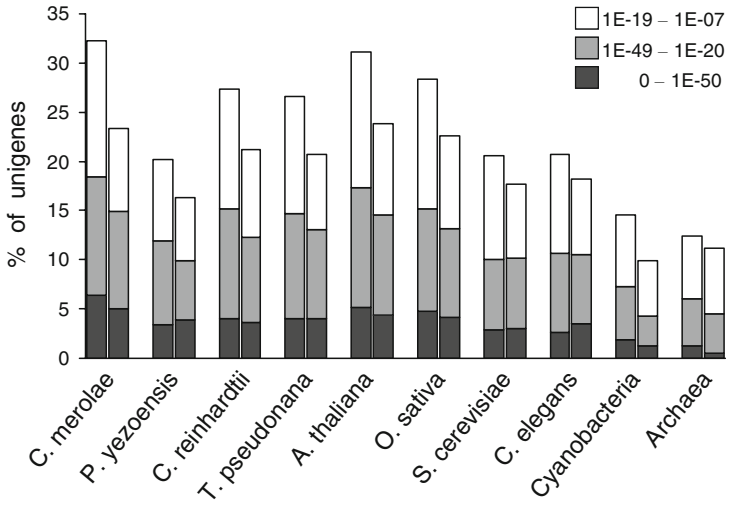

Figure 2. BLASTX similarity BLASTX similarity of *Porphyridium* sp. (first bar of each pair of bar diagrams) and *Dixoniella grisea* unigenes (second bar) to various genomes (E-value 1E-07).

than to the phylogenetically more distant eukaryotes (yeast and nematode), indicating the red algae are more related to eukaryotes than to the prokaryotes.

The Arad group compared 2,062 unigenes of *Porphyridium* sp. to genome-derived proteins of *C. merolae* (5,013), *C. reinhardtii* (19,832), and *T. pseudonana* (11,397) (Fig. 3a). BLASTX (>1e-07) analysis showed that while most of the *Porphyridium* sp. unigenes (1,288; 62%) did not exhibit similarity to any of three genomes analyzed, 412 unigenes (20%) that did have similarity, are common to all three genomes compared. Similarly, when *T. pseudonana* was replaced by cyanobacteria in the Venn diagram (Fig. 3b), the similarity of *Porphyridium* sp. to all the species tested was further reduced to 218 (11%), and the number of unigenes for which there was no similarity increased to 1,301 (63%), showing that *Porphyridium* sp. is closer to eukaryotic algae than to prokaryotic cyanobacteria. As expected, there was a higher similarity between *Porphyridium* sp. and *C. merolae* – both belonging to the Rhodophyta [despite the smaller number of genome-deduced proteins (5,013) for *C. merolae*] – than to *C. reinhardtii* (19,832) and *T. pseudonana* (11,397). These findings, together with the high similarity between *Porphyridium* sp. and *C. reinhardtii* and *A. thaliana* (Fig. 2), support the viewpoint of various authors (Burger et al., 1999; Moreira et al., 2000; Ragan and Gutell, 1995) that the red algae and Viridaeplantae (green algae and embryophytes) are sister groups.

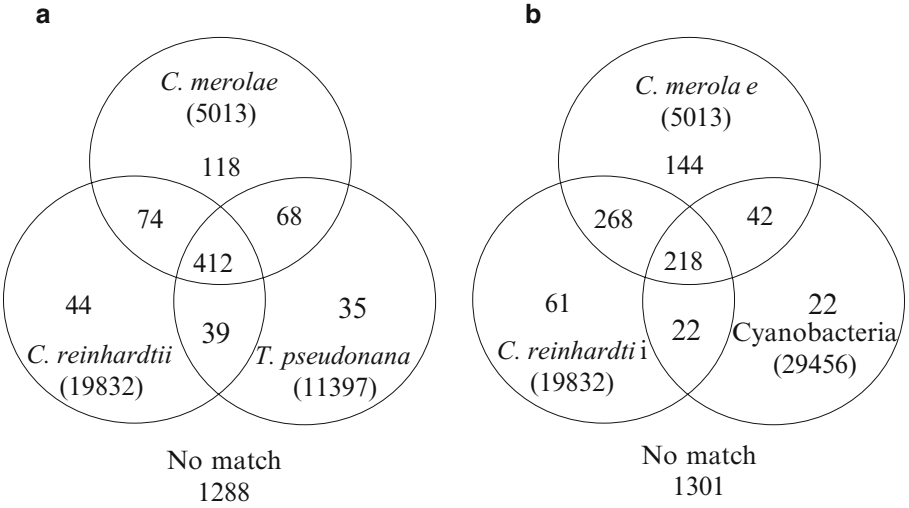

Figure 3. Venn diagram similarity Venn diagrams showing the similarity of *Porphyridium* sp. proteins to homologs in other organisms (BLAST e-value cutoff >100). Distribution of 2,062 *Porphyridium* sp. derived proteins with homology to proteins from the red alga *Cyanidioschyzon merolae* (**a,b**), the green alga *Chlamydomonas reinhardtii* (**a,b**), the diatom *Thalassiosira pseudonana* (**a**), or cyanobacteria (**b**). Numbers in parentheses show the number of proteins used in the analysis. Numbers below the circles indicate the number of *Porphyridium* sp. proteins with no homology to the examined proteomes.

4. Genetic Engineering in Red Microalgae

4.1. PERSPECTIVES: ACHIEVEMENTS AND DIFFICULTIES

The powerful tools of genetic engineering, like the technologies for gene transfer, gene knockout, or gene replacement, had already been established for bacteria, animals, and plants as long as two decades ago. Since then, progress in developing molecular tools has also been made for some eukaryotic algae, especially species of green algae and diatom families (Apt et al., 1996; Falciatore et al., 1999; Fletcher et al., 2007; Griesbeck et al., 2006; Galvan et al., 2007; Gruber et al., 2007; Hallman, 2007; Purton, 2007; Zaslavskaia et al., 2000). To date, several microalgal species have been successfully transformed by using a variety of strategies and vector components – different promoter and selection/reporter genes. However, the effort to generate transformation systems for red microalgae has met with little success, with the exception of a report for the *Porphyridium* sp. chloroplast (Lapidot et al., 2002) and one for the *C. merolae* nucleus (Minoda et al., 2004).

Since reproduction in red microalgae is asexual, the absence of well-established gene transfer technologies for red microalgae constitutes a bottleneck in efforts to reveal the metabolic pathways of their unique biochemicals, to resolve basic and evolotionary questions, and to exploit their biotechnological potential. The development of an easy-to-handle transformation system for red microalgae must overcome the main obstacle of the lack of a suitable selection marker that can differentiate between transformed and nontransformed cells in an axenic culture. One of the problems in finding a suitable marker is that most red microalgae are not sensitive to a broad spectrum of commonly used antibiotics, like kanamycin, ampicillin, and spectinomycin (for example, see Table 2).

Table 2. Effect of antibiotics and inhibitors on the growth of *Porphyridium* sp.

Compound	Dose	Growth
Paromomycin	200 μg/ml	++
Neomycin	150 μg/ml	++
G418	2 mg/ml	+
Phleomycin	10 μg/ml	++
Hygromycin	1 mg/ml	++
Lincomycin	300 μg/ml	++
Chloramphenicol	200 μg/ml	+
	500 μg/ml	–
Spectinomycin	300 μg/ml	++
Kanamycin	700 μg/ml	++
Methotrexate	80 μg/ml	++
Cycloheximide	5 μM	–
Zeocin™	4–8 μg/ml	–

++, mean growth rate equal to that of the wild-type.
+, mean growth rate slightly slower than that of the wild-type.
–, no growth.

The first step in the efforts of the Arad laboratory to develop the biotechnology of red microalgae was the isolation of various resistant and phenotypic mutants. The first mutant to be isolated in red microalgae – designated SMR-1 – was a mutant resistant to the herbicide sulfometuron methyl (SMM), which targets acetohydroxy acid synthase (AHAS) (van Moppes et al., 1989). Various resistant and phenotypic mutants, especially mutants of *Porphyridium* sp., were subsequently generated (Arad et al., 1993a, 1994; Sivan and Arad, 1993, 1995). In parallel, a technology for protoplast fusion was developed (Sivan and Arad, 1998; Sivan et al., 1995); this technology has the dual advantage of combining multigenetic traits and generating novel cellular entities that combine nuclei and cytoplasmic organelles originating from different strains. The technology is based on intraspecific transfer of resistance to different substances between two different resistant mutants. Yet, despite its promise, it appears that this technology has not fulfilled its potential.

4.1.1. Chloroplast and Nuclear Gene Transformation

Chloroplast transformation was the first gene transfer technology to be reported for *Porphyridium* sp. The availability of the SMM-resistant *Porphyridium* sp. mutant enabled the cloning of an endogenous SMM-resistant *AHAS* form (Lapidot et al., 1999). Since *AHAS* is encoded in the chloroplast of red algae – in contrast to green algae and plants where it is encoded in the nucleus – the transformation system based on the resistant *AHAS* form targets the chloroplast genome via homologous recombination without the need for a promoter sequence (which was not available at that time) (Lapidot et al., 2002).

The group of Arad has recently developed a transformation technology targeting the nuclear genome of *Porphyridium* sp. on the basis of cell growth sensitivity to the relatively new drug Zeocin™. A bacterial resistant gene, designated *Sh-ble*, was set in a vector cassette plasmid flanked by the cauliflower mosaic virus CaMV 35S promoter, which had previously been developed for plant transformation and had also been used in transformation of eukaryotic algae (Hawkins and Nakamura, 1999; Jarvis and Brown, 1991) and its poly A site sequence. Transformed colonies appeared on agar plates after agitating the algal cells with the vector plasmid in the presence of glass beads. The cells became competent after a dark period of at least 12 h and allowed incorporation of the foreign DNA molecule. Transformed cultures of *Porphyridium* sp., in the presence of up to 15 ng of Zeocin, exhibited the same growth pattern as a wild-type culture in the absence of the drug (Fig. 4). The presence of the foreign DNA in the cells was verified by PCR on several independent transformed colonies and by Southern blot analysis (Plesser, 2004).

The transformation of the red microalga *C. merolae* was recently reported by Minoda et al. (2004). In that work, a spontaneous mutant resistant to 5 fluoroortic acid (FOA), which is UMP synthase deficient, was isolated. The uracil auxotrophic mutant cells were then restored by introducing the wild-type UMP

Figure 4. Transformed algal cultures Transformed *Porphyridium* sp. cultures after 17 days of growth in artificial sea water (ASW) containing 4 μg/ml of Zeocin. The control wild-type was transformed without vector.

synthase gene into the cells, which suggests DNA transformation by homologous recombination to the nuclear genome. The introduction of the foreign DNA was performed without the addition of a promoter or poly A site sequences.

4.1.2. Red Microalgae as Cell Factories: Difficulties and Potential

Since red microalgae have the ability to grow rapidly under controlled conditions utilizing sunlight as the energy source for photosynthesis, they can be used as cell factories for the production of valuable proteins. The recent development of gene transfer methods for an increasing number of microalgal species has opened new opportunities to harness microalgae cells for biotechnological applications as high-throughput expression systems for a variety of proteins, including enzymes, antibodies, growth factors, and drugs (Hawkins and Nakamura, 1999; Mayfield et al., 2003).

Moreover, since red microalgae are edible and have additive value as a health food (Dvir et al., 2000, 1996), genetically engineered microalgal cells may be used as oral vaccines. The algae produce the antigenic protein and may serve as the vehicle for vaccine delivery as well. Edible vaccines of this type are produced by introducing selected gene(s) that code for an antigen of interest into the edible organism. Such vaccines are administered orally, providing a low-cost, easy-to-administer alternative to parenteral delivery, and thereby the possibility of quick mass immunization. In addition, the need for trained personnel, refrigeration, and shipping expenses are reduced.

In a study using ovalbumin as the model antigen, the Arad group has already demonstrated the potential of a transgenic red microalga species as an oral vaccine. The ovalbumin gene was introduced into the *Porphyridium* sp. cell

via a nuclear transformation system. The highest expression level was calculated to be ~24 μg of recombinant ovalbumin per 1 g of biomass. In that study, mice were fed 5 g of dry transgenic algae expressing ovalbumin for one day per week. After four weeks of feeding, significant titers of anti-ovalbumin antibodies, mostly IgA and IgG isotypes, were detected in the blood (Manandhar-Shrestha, 2007).

5. Concluding Remarks

The development of a biotechnological process – taking an idea from the lab to the market – is exemplified in the ongoing R&D program of the Arad laboratory on red microalgae. The multidisciplinary development – integrating biological and molecular studies with engineering – has followed a long, but very fascinating, path to its present stage: The technology for large-scale production of the algal biomass and sulfated polysaccharides has been running successfully for a number of years on pilot and industrial scales; the sulfated polysaccharides have already found applications in cosmetic products, and other applications are in advanced stages of development. The future is taking us towards further exploiting the polysaccharide – a novel molecule with additional potential applications – and towards unraveling the enormous potential that lies in the genome of the red microalga, a subject that we have touched only on the surface.

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RED ALGAL GENOMICS: A SYNOPSIS

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1. Introduction

The red algae (or Rhodophyta) are an ancient and diversified group of photoautotrophic organisms. A 1,200-million-year-old fossil has been assigned to *Bangiomorpha pubescens*, a *Bangia*-like fossil suggesting sexual differentiation (Butterfield, 2000). Most rhodophytes inhabit marine environments (98%), but many well-known taxa are from freshwater habitats and acidic hot springs. Red algae have also been reported from tropical rainforests as members of the subaerial community (Gurgel and Lopez-Bautista, 2007). Their sizes range from unicellular microscopic forms to macroalgal species that are several feet in length. In aquatic environments, they are found as members of the planktonic and benthonic communities. Rhodophytan life cycles are highly contrasting. They include simple life cycles characterized by binary cell division and complex triphasic, haplo-diplobiontic life cycles, with one haploid (gametophytic) and two diploid (tetrasporophytic and carposporophytic) generations. The latter life cycle can be either isomorphic or heteromorphic. This is dependent on whether or not gametophytes and tetrasporophytes are morphologically similar or dissimilar, respectively. Rhodophyta share many biochemical and ultrastructural features with other algal groups. However, they are often characterized by a unique set of features such as the absolute lack of flagella and centrioles, presence of phycobilisomes and unstacked thylakoids in the chloroplast, absence of parenchyma, and presence of pit-connections between cells. Rhodophyta are characteristically red in color, but other shades of green, brown, and purple are common. Photosynthetic pigments include chlorophyll *a*, which is accompanied by accessory pigments or phycobiliproteins. These phycobiliproteins are responsible for the alga's red coloration. They include water-soluble pigments such as phycoerythrin (red), phycocyanin (blue), and allophycocyanin (blue-greenish) (Grossman et al., 1993). These red and blue pigments are associated into phycobilisomes, a light-harvesting complex, on the surface of thylakoids. In this report, a summary is presented of genomic studies targeting this algal group, the Rhodophyta, with suggestions for future research in phylogenomics.

Red algae systematics is an exciting and dynamic field, because of recent advancement in molecular biology, computational technology, and phylogenetics. New classification systems and new taxa descriptions are frequent in recent systematic literature. The two most recent current systems of classification were

proposed by Saunders and Hommersand (2004) and Yoon et al, (2006). These systems recognize the red algae as one of the earliest divergent groups in the Plant Kingdom, the monophyly of Florideophyceae, a redefined Bangiophyceae *sensu stricto*, and the sister position of Cyanidiales to the rest of the red algae. Both systems recognize the class level of the latter group, as Cyanidiophyceae, a level previously revived by Seckbach (1999). This intriguing group of ancient red algae is found inhabiting acidic hot springs.

During the last 15 years, rhodophytan systematics has made significant progress due to the advent of molecular-based analyses (Gurgel and Lopez-Bautista, 2007). The two most commonly used genes are the chloroplast-encoded *rbc*L large subunit of the ribulose 1,5-bisphosphate carboxlase/oxygenase enzyme and the nuclear-encoded ribosomal cistron encoding 18S, ITS1, 5.8S, ITS2, and the 28S rDNA regions (Freshwater et al., 1994; Harper and Saunders, 2001; Hommersand et al., 2006). The latest systematic account by Yoon et al. (2006), using the *psa*A (PSI P700 chl *a* apoprotein A1) and *rbc*L coding regions resulted in the recognition of seven major lineages in Rhodophyta. These authors proposed Rhodophyta to be classified in two new subphyla, the Cyanidiophytina with a single class Cyanidiophyceae and the Rhodophytina, which include six classes: Bangiophyceae, Compspogonophyceae, Florideophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae. Recently, a new order, Rufusiales, has been inferred as a potential sister group to Stylonematophyceae (Zuccarello et al., 2008). A member of this order, *Rufusia pilicola*, is found in a rather unusual habitat, sloth fur.

Representatives of red algae are used as an economic resource for humans. Diverse industries based on red seaweeds account several billions of dollars per year. Most of these industries are related to human consumption in the form of aquaculture and phycocolloid production (Gurgel and Lopez-Bautista, 2007). Red seaweeds are used as food. This is well represented by the processed food nori (*Porphyra* spp.) and many other red algae. The phycocolloid industry harvest many different kinds of red seaweeds for their highly valuable gels. These seaweeds include carrageenophytes (*Chondrus crispus* and other Gigartinaleans) and agarophytes (i.e., species of *Gracilaria* and *Gelidium*). From a negative point of view, some red algae have been deemed as invasive species. Recently, reports of direct or indirect human introductions of nonindigenous red algae have accumulated; these reports include areas of Australia, Brazil, Europe, and USA. Negative effects from these invasive species include losses in native biodiversity, detrimental effects to the fishing industry, and a loss of recreational activities (Ruiz et al., 2000; Ribera-Siguan, 2003).

The importance of red algae is undeniable. From the biological point of view, they are amid the most ancient eukaryotic photobionts on our planet. A plethora of molecular phylogenies have accumulated in the last decade. This has resulted in a modern rhodophytan classification system that reflects evolutionary relationships. With the advancement of new molecular biology techniques, the development of faster computer systems, and increasingly more affordable DNA

sequencing, the analysis of complete red algal genomes has become a tangible and desirable goal (Weber et al., 2007).

2. Nuclear Genomes of Red Algae

In a series of recent publications (Matsuzaki et al., 2004; Misumi et al., 2005; Nozaki et al., 2007), a 100%-complete nuclear genome sequence for the rhodophytan *Cyanidioschyzon merolae* was elucidated. This is the first published complete nuclear genome known for a red alga. More information on *C. merolae* Genome Project can be found at http://merolae.biol.s.u-tokyo.ac.jp/. The nuclear genome of *C. merolae* consists of 16,546,747 bp and 4,775 protein-coding genes were identified (Nozaki et al., 2007). Matsuzaki et al., 2004; Misumi et al., 2005; and Nozaki et al., 2007 have highlighted some notorious and interesting features for this microscopic rhodophyte, which lives in extreme environments. All histones genes in *C. merolae* are located on a single chromosome forming the most compact gene cluster ever reported. Telomeres termini in *C. merolae* are unique in having telomere repeats of AATGGGGGG at all chromosomal ends. Most plants have telomere repeats of TTTAGGG. Transposable elements, 26 class I elements (retrotransposons) and 8 class II elements (transposons), only account for 0.7% of the *C. merolae* genome. This is an extremely low value when compared with other genomes. Only three copies of the rRNA gene units and a low number of genes with introns (0.5%) were found in *C. merolae*. These set of characteristics have prompted these authors (Nozaki et al., 2007) to emphasize that the nuclear genome of *C. merolae* is not only unique but also "constitutes the simplest set of genomic features found in any nonsymbiotic eukaryote yet studied." These attributes are an example of a reductive evolution in a very small eukaryote organism. Furthermore, these features were considered plesiomorphic (ancestral) characters of the nuclear genome. As an alternative explanation, Nozaki et al. (2007) interpreted these genomic features as adaptations to the extreme environments.

Another ongoing project of a Cyanidiophyceae, *Galdieria sulphuraria*, has become available for comparison (Weber et al., 2004, 2007; Barbier et al., 2005). The *G. sulphuraria* genome project can be accessed at http://genomics.msu.edu/galdieria. Information on nuclear genome size for species of *Galdieria* have been reported between 10 and 17 Mbp (Barbier et al., 2005) and thus in the range with *C. merolae*. Although found in similar thermoacidophilic habitats, *C. merolae* and *G. sulphuraria* have various differences. While *C. merolae* is an aquatic obligate photoautotroph, reproducing by binary fission, and lacking a rigid cell wall, *G. sulphuraria* is a metabolically flexible (photoautotroph, heterotroph, and mixotroph), can live endolithically, reproducing by endospores, and contains a rigid cell wall (Oesterhelt et al., 2008; Barbier et al., 2005). Based on a comparative analyses between the *C. merolae* and >70% of the genome of *G. sulphuraria*, Barbier et al. (2005) concluded that over 30% of *G. sulphuraria* sequences have no similarity with genes from *C. merolae.* Furthermore, the same study estimated

that *G. sulphuraria* contain more introns, membrane transporters, and enzymes for carbohydrate metabolism than *C. merolae.* Some of these gene features are most likely attributed to the presence of a rigid cell wall and mixotrophic abilities demonstrated by *G. sulphuraria* (Oesterhelt et al., 2008; Barbier et al., 2005).

3. Chloroplast Genomes of Red Algae

The number of studies on chloroplast genome sequences for red algae includes more taxa than that for nuclear genomes. This fact maybe, partially, due to the smaller size of the chloroplast genome, which results in faster sequencing time and reduced fees. It could also be due to the wealth of valuable phylogenetic information revealed by the structure of the chloroplast genome. In green algae and land plants, for example, recent analyses of the architecture of the chloroplast genome (Pombert et al., 2005; Turmel et al., 2006; Pombert et al., 2006; Turmel et al., 2007) have challenged our previous understanding of evolution in Viridiplantae. In these remarkable studies, phylogenetic analyses of several genes as well as gene and intron content, gene order, and insertion/deletion of coding regions have uncovered the green algal ancestry of land plants. In Rhodophyta, studies on the architecture of the chloroplast genome have been reported in three of the seven classes (Table 1), which include two Cyanidiophyceae *C. merolae* (Ohta et al., 2003) and *Cyanidium caldarium* (Glöckner et al., 2000), one Bangiophyceae *Porphyra purpurea* (Reith and Munholland, 1993, 1995), and the Florideophyceae *Gracilaria tenuistipitata* var. *liui* (Hagopian et al., 2004).

In photosynthetic eukaryotes, chloroplast genome sizes range from 35 to 200 kb (Hagopian et al., 2004). Chloroplast genome features from rhodophytans are given in Table 1. These red algal chloroplast genomes are in the higher size range but show similar amounts of bp among them from 150 to 191 kb. Other rhodophytan plastid genomes also fall into the same range such as *Porphyra yezoensis* 185 kb (Shivji, 1991), *Griffithsia pacifica* 178 kb (Li and Cattolico, 1987), *C. crispus* 173 kb, and *Antithamnion* sp. 180 kb (Simpson and Stern, 2002). Surprisingly, rhodophytans lack introns. In contrast, the green lineage is known

Table 1. Chloroplast genome characteristics of red algae.

	Class: Cyanidiophyceae		**Class: Bangiophyceae**	**Class: Florideophyceae**
	C. merolae	***C. caldarium***	***P. purpurea***	***G. tenuistipitata***
Length (bp)	149,987	164,921	191,028	183,883
Genes	243	232	251	238
G + C (%)	37.6	32.7	33	29.1
rRNA operons	1	1	2	1
Introns	None	None	None	None
Reference	Ohta et al. (2003)	Glöckner et al. (2000)	Reith and Munholland (1995)	Hagopian et al. (2004)

to have many chloroplast introns. Reith and Munholland (1993) argued that the absence of introns might represent an ancestral character trait, since a similar situation is found in eubacteria. In terms of number of chloroplast rRNA operons, most red algae contain only one copy (including also *G. pacifica* [Shivji et al., 1992] and *C. crispus* [Leblanc et al., 1995a, b]); *P. purpurea* is the lone exception with a reported rRNA operon duplication (Reith and Munholland, 1995). Because of the sister relationship between the Bangiophyceae (*Porphyra*) and the Florideophyceans (*Gracilaria, Griffithsia,* and *Chondrus*) Hagopian et al. (2004) suggested that *Porphyra's* rDNA direct repeat was a condition that was lost secondarily in Florideophyceae.

Red algal chloroplasts have roughly the same number of genes. The range of genes in red algae fluctuates between 232 and 251. However, when the number of genes in the rhodophytan chloroplast genomes is compared with those from the green algae, a far greater distance is evident. Green algae and land plants have been shown to have only between 110 and 118 genes in the chloroplast (Hagopian et al., 2004). In this regard, we can consider the chloroplast genome of red plastids as having twice the number of genes than green plastids. Most of the genes found in the green lineages are related to photosynthesis and gene expressions. In contrast, most of the genes found in the red algal lineage are related to ribosomal proteins and photosynthesis components (Ohta et al., 2003). Chloroplast genomes of red algae have been shown to contain the most ancient repertoire of genes among the photosynthetic eukaryotes (Hagopian et al., 2004). This genome condensation is particularly remarkable in the Cyanidiophyceae, *C. merolae,* with up to 40% of its protein genes overlapped (Ohta et al., 2003), and in both, *C. merolae* and *C. caldarium,* where the median intergenic distance is shorter (14 and 60 bp, respectively) than other red algae (i.e., *P. purpurea* with 100 bp; Ohta et al., 2003). Chloroplast genomes from Cyanidiophyceae, *C. caldarium* and *C. merolae*, share a significant number of genes (Ohta et al., 2003). Many notable features of the red algal chloroplast genome can be interpreted as a result of their extremophilic characteristics (thermoacidophilic habitats). In these habitats, similar evolutionary selection pressures may help to maintain similar chloroplast genes (Glöckner et al., 2000).

Plastid genome evolution has been an exciting field since the publication of Mereschkowsky's hypothesis (1905) that explained for the first time that plastids evolved from endosymbiotic cyanobacteria (Raven and Allen, 2003). From this initial primary endosymbiotic event, three lineages evolved – Glaucocystophyta, Chlorophyta, and Rhodophyta. The origin of plastids from a single primary endosymbiosis that involved, a eukaryote and a cyanobacterium has been a common understanding (Keeling, 2004). However, recent investigations on the freshwater amoeba *Paulinella chromatophora* with a second and more recent primary endosymbiosis are challenging this concept (Rodriguez-Ezpeleta and Philippe, 2006). The green algae or Chlorophyta, one of the primary plastid lineages, in turn became the source for plastids via a secondary endosymbiosis event. This secondary green lineage includes the Euglenophyta and the Chlororachniophyta.

The plastids of red algae or Rhodophyta, a primary plastid lineage, through secondary endosymbiosis generated a diverse group of algal groups. This secondary red lineage includes the Cryptophyta, Heterokontophyta, Haptophyta, and probably some Dinoflagellates (Dinophyta) (Delwiche, 1999). One remarkable difference between these plastids and the free-living cyanobacteria is the reduction in both genome size and gene content, in the plastid genome. For example, plastid genomes encode for about 5–10% of proteins when compared with free-living cyanobacteria (Ravi et al., 2008). Genome size of the cyanobacterium *Synechocystis* PCC 6803 contains 3,573,470 bp and ca. 3,168 genes (Kaneko et al., 1996) while in red algae the genome size range is between 150 and 191 kb with a gene content fluctuating between 232 and 251 (Table 1). The reduction of the plastid genome size has been explained by implicating three mechanisms – loss of plastid genes lacking selective advantage, substitution of plastid genes by pre-existent nuclear genes, and transfer of plastid genes to the nuclear genome (Delwiche, 1999).

4. Mitochondrial Genomes of Red Algae

Studies on complete mitochondrial genomes of red algae are not as numerous as chloroplast genomes. In green algae, mitochondrial genomes have been analyzed for evolutionary relationships (Nedelcu et al., 2000; Pombert et al., 2006). In these chlorophytan lineages, two different mitochondrial genome types have been described – a reduced-derived and an ancestral type (Nedelcu et al., 2000). In other studies, comparative analyses of mitochondrial genomes are challenging their higher rank relationships among green algal lineages (Pombert et al., 2006). In red algae, complete analyses of the architecture of the mitochondrial genome have been reported (Table 2) for three (out of seven) classes of rhodophyta: the Cyanidiophycean *C. merolae* (Ohta et al., 1998), the Bangiophycean *P. purpurea* (Burger et al., 1999), and the Florideophycean *C. crispus* (Leblanc et al., 1995b).

In general, mitochondrial genome sizes fall into two extremes (Leblanc et al., 1995b). There are small mitochondrial genomes, which are usually found in animals. These genomes range between 14 and 42 kb, they are uniform in architecture, and are extremely compact. In contrast, green plant mitochondrial genomes are larger, more complex, and range in size from 200 to 2,400 kb (Leblanc et al., 1997).

Table 2. Mitochondrial genome characteristics of red algae.

	Class: Cyanidiophyceae	**Class: Bangiophyceae**	**Class: Florideophyceae**
	C. merolae	***P. purpurea***	***C. crispus***
Length (bp)	32,211	36,753	25,836
Genes	34	57	51
G + C (%)	27.2	33.5	27.9
Reference	Ohta et al. (1998)	Burger et al. (1999)	Leblanc et al. (1995b)

In Rhodophyta, mitochondrial genomes are small with a range in size from 25.8 to 36.7 kb. Further reports indicate a similar range in *Gracilariopsis lemaneiformis (40 kb), G. pacifica (25–28 kb), and C. caldarium* (33 kb) (Leblanc et al., 1997). Rhodophytan mitochondrial genomes are considerably smaller than green plants. However, rhodophytans have a genomic size in a similar range to the green algae (15.8–55.3 kb) (Nedelcu et al., 2000). Although Turmel et al. (2007) discovered in *Chlorokybus atmophyticus*, an early divergent clade of the charophycean lineage, an unexpectedly large (201,763 bp) mitochondrial genome. Analyses on *C. merolae*'s mitochondrial genome highlighted the similarities between its mitochondrial genome and those from animals and plants (Ohta et al., 1998). Compactness of genome in *C. merolae*, absence of introns, the encoding for only 34 proteins, presence of short intergenic spacers, and a high coding density explain in part the reduced size of *C. merolae* mitochondrial genome (Ohta et al., 1998). Small compact genomes are also reported for *P. purpurea* (Burger et al., 1999). Only 9% of its genome is without detectable coding content. Similarly, *C. crispus* (Leblanc et al., 1995b) includes a high coding density with only 4.8% of noncoding regions reported. Only one group II intron has been reported for *C. crispus* (Leblanc et al., 1995b). Two group II introns interrupt the LSU rRNA coding region for *P. purpurea* (Burger et al., 1999). According to Burger et al. (1999), mitochondrial genomes from *P. purpurea* and *C. crispus* are almost identical in gene content and order. Although the universal genetic code is used in mitochondrial genomes of land plants and some chlorophytes and heterokontophytes, there are a multitude of examples that deviate from the universal code (Leblanc et al., 1997). One example is the modification of the termination code UGA to tryptophan. This is the case for *C. crispus* (Leblanc et al., 1995b). However, this modification is absent in *C. merolae* or in *C. caldarium* (Ohta et al., 1998). These data seem to indicate that the stop UGA codon was the ancestral character state in Rhodophyta. Therefore, the UGA tryptophan codon represents an autoapomorphy for the *C. crispus* lineage (Ohta et al., 1998; Leblanc et al., 1995b).

There is a consensus in the scientific community that mitochondria have evolved through an endosymbiotic process (analogous to plastid evolution). This process involved a unicellular phagotrophic eukaryote engulfing prokaryotic organisms that eventually became (through endosymbiosis) the eukaryotic organelles. This view of a nucleus-bearing amitochondriate cell as the original eukaryote host cell has been debated. An alternate scenario that involves a prokaryotic host cell has been proposed (Embley and Martin, 2006). The endosymbiotic origin of the mitochondria can be traced back to eubacterial lineages, in particular to α-proteobacteria (Leblanc et al., 1997; Ohta et al., 1998). Although monophyletic in origin, the mitochondrial genome exhibits a wide diversity in gene size, order, and content in extant taxa. Approximately half of the mitochondrial gene set was lost in rhodophytes after the divergence from a common ancestor shared with chlorophytes (Burger et al., 1999). An evolutionary explanation for the rhodophytan genome compactness is that red algae lost genes at faster rates than green algae (Ohta et al., 1998). Mitochondrial genome diversity

is exemplified by the two extremes where one mitochondrial genome is larger and the other is smaller (Leblanc et al., 1997). The addition of newly sequenced mitochondrial genomes may help one to explain the aforementioned extreme size range. According to Leblanc et al. (1997), "paralogous evolution resulting from similar evolutionary constraints and strategies" are yet to be discovered.

5. Conclusions

The Rhodophyta are an ancient group of eukaryotes with over 6,000 species and can be found in most regions in the planet. They are considered the sister group to green algae and land plants. Over the last 15 years, there has been an increase in available sequence data for the analysis of red algal genomes. Perhaps, owing to their obvious presence and economical importance, green plants have been the common sources for genomic research other than red algae (Reyes-Prieto et al., 2006). Recommendations for future algal genomic research have been proposed. However, there are several criteria that need to be met before an algal candidate is selected. Waaland et al. (2004) highlighted the absence of major efforts to complete nuclear genome sequence from a macroalga. The only completed nuclear genome in Rhodophyta is that of *C. merolae*, a microscopic alga (Nozaki et al., 2007). Among the criteria to select algal candidates for genomic sequencing, Waaland et al. (2004) and Grossman (2005) proposed: growth in a defined medium, defined sexual life cycle, economic importance, evolutionary interest, ecological importance, uninucleate cells, and established background of scientific information. Of course, the researchers must also be both knowledgeable and belonging to a well-organized community of scientists. The data presented in this synopsis point out that the rhodophytans already investigated fall under the criteria of highly significant taxa for the evolution and ecology of extremophiles (Cyanidiophyceae) as well as for their economic importance (*Porphyra* spp., *Gracilaria* spp., *Chondrus crispus*, and more recently *Gracilaria changii* (Teo et al., 2007)). With the recent emergence of phylogenomics, a new area of research is becoming increasingly important in red algal genomics (Eisen and Fraser, 2003). Phylogenomics is a new field where, as described by Reyes-Prieto et al. (2006), genomics (the study of the function and structure of genes and genomes) has intersected with molecular phylogenetics (the study of the hierarchical evolutionary relationships among organisms, their genes, and genomes). There are some caveats while working with genome-scale phylogenies (as pointed out by Rodriguez-Ezpeleta et al., 2007). However, the application of this new field to molecular systematics is highly promising. A recent study by Li et al. (2006) has outlined a phylogenomic pipeline and discovered a significant endosymbiotic gene transfer from red algal genes shared with chromalveolates (see Blackwell, 2009 for discussion of names) and suggesting a monophyletic origin for some of the taxa under study. It is clear that phylogenomic research will be an ever increasing field in algal systematics. Thus, there is a need, as suggested by Waaland et al. (2004)

and pointed out by Grossman (2005), to select candidates for genomic studies that are "positioned at important evolutionary branchpoints." Yoon et al. (2006) defined the major lineages of red algae as consisting of seven classes. Our current knowledge of complete genomes of rhodophyta is circumscribed to only one class for a nuclear genome (Cyanidiophyceae; Nozaki et al., 2007). Chloroplast and mitochondrial genomes are known only for three different red algal classes (Cyanidiophyceae, Bangiophyceae, and Florideophyceae). Overall, only one rhodophytan genome, *C. merolae,* has been completely sequenced (nuclear, chloroplast, and mitochondrial) and accounts for a total of 16,728,945 bp for its entire genome (Nozaki et al., 2007). Several classes in the rhodophytan system (Yoon et al., 2006) are still lacking information for phylogenomic comparison including the Compsopogonophyceae, the Porphyridiophyceae, and the Rhodellophyceae. This phylogenomic information may provide a clearer vision of the systematics of Rhodophyta to understand the evolutionary history of this ancient group of red algae.

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7. References

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BANGIOPHYTES: FROM ONE CLASS TO SIX; WHERE DO WE GO FROM HERE?

Moving the Bangiophytes into the Genomic Age

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1. Introduction

The Rhodophyta represent a distinct eukaryotic lineage (Gabrielson et al., 1990; Freshwater et al., 1994; Ragan et al., 1994; Stiller and Hall, 1997; Yoon et al., 2006b) that shares the most recent common ancestry with the green algae (Burger et al., 1999; Baldauf et al., 2000; Moreira et al., 2000; Rodríguez-Ezpeleta et al., 2005). Members of this phylum share a set of characteristics that do not occur together in other eukaryotes. For example, this group lacks flagellated stages and basal bodies, possesses a two-membrane-bound plastid with unstacked thylakoids but lacking chlorophyll *b* or *c*, and contains photosynthetic reserves stored as floridean starch (Gantt et al., 1986; Garbary and Gabrielson, 1990; Broadwater and Scott, 1994; Bhattacharya and Medlin, 1995). Traditionally, the members of the Rhodophyta have been divided into two groups (Table 1) – the "florideophytes" and the "bangiophytes," with the latter name used to identify the early diverging red algae from which the former group has evolved. Gabrielson et al. (1985) recognized the red algae as consisting of two subclasses – the Bangiophycidae and the Florideophycidae, within one class, the Rhodophyceae. This proposal differs from that presented by Van den Hoek et al. (1995) in which two classes, the Bangiophyceae and Florideophyceae, are recognized. Saunders and Hommersand (2004) recognized four classes: the Bangiophyceae, Composopogonophyceae, Florideophyceae, and Rhodellophyceae, the last of which contained two clades of the paraphyletic order Porphyridiales, and the Stylonematales. In addition, these authors suggested that members of the Cyanidiales belong to their own phylum, the Cyanidiophyta. More recently, Yoon et al. (2006b) recognized seven classes within one phylum and included members of the Cyanidiales within this phylum. For the purpose of simplicity, we will follow the most recent taxonomic assessment in our discussion of these enigmatic groups (Table 1).

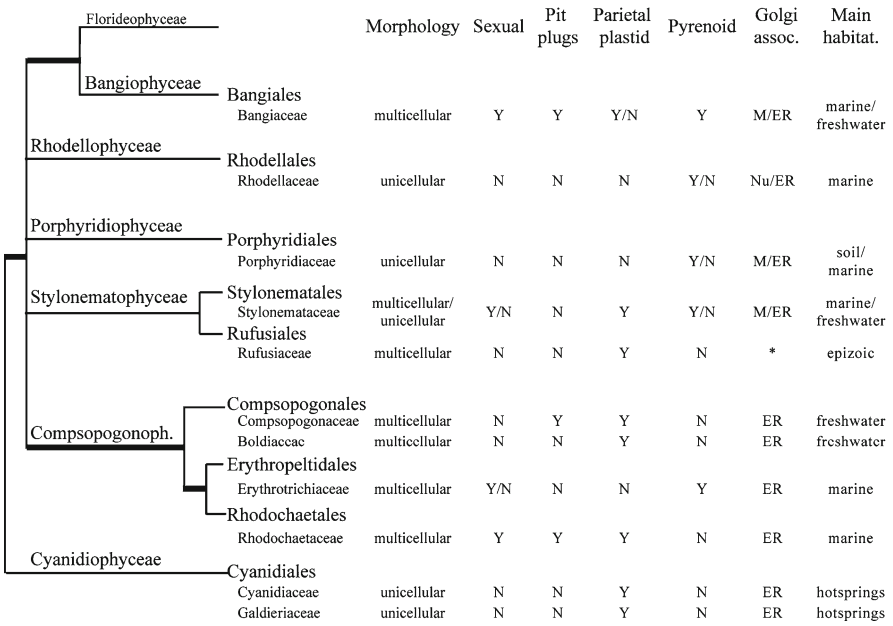
The Florideophyceae includes morphologically complex red algae in orders, such as the Gigartinales and the Ceramiales, and is generally considered to be a

Table 1. Classification of the rhodophyta at the phylum, subphylum, class, and subclass (only Garbary et al., 1980) levels.

Garbary et al. (1980)	Van den Hoek et al. (1995)	Saunders and Hommersand (2004)	Yoon et al. (2006b)
Rhodophyceae	Rhodophyta	Rhodophyta	Rhodophyta
		Eurhodophytina	Rhodophytina
Florideophycidae	Florideophyceae	Florideophyceae	Florideophyceae
Bangiophycidae	Bangiophyceae	Bangiophyceae	Bangiophyceae
		Rhodellophytina	
		Rhodellophyceae	Rhodellophyceae
			Porphyrdiophyceae
			Stylonemat ophyceae
		Metarhodophytina	
		Compsopogonophyceae	Compsopogonophyceae
		Cyanidiophyta	Cyanidiophytina
		Cyanidiophyceae	Cyanidiophyceae

monophyletic group (Garbary and Gabrielson, 1990; Ragan et al., 1994; Freshwater et al., 1994; Saunders and Kraft, 1997) that is a sister group to the Bangiophyceae s.s. (sensu stricto) (Müller et al., 2001; Saunders and Hommersand, 2004; Yoon et al., 2006b). The Bangiophyceae s.l. (sensu lato) is considered to be the ancestral pool of red algae and range from simple unicells to filaments (uniseriate or multiseriate; branched or unbranched), saccate or sheet-like thalli (monostromatic or distromatic, Garbary et al., 1980). The typically single plastid within this group is axial and stellate with a large pyrenoid (e.g., Porphyridiophyceae). However, some taxa have a cup-shaped plastid (Cyanidiophyceae), or a complex, lobed plastid +/– pyrenoids (Compsopogonophyceae) (Fig. 1; Gantt et al., 1986; Broadwater and Scott, 1994; West et al., 2007a). Pit connections are rare but have been noted in some groups, including the conchocelis stage in members of the Bangiophyceae s.s. and *Rhodochaete* (Ueki et al., 2008 and references therein). Sexual reproduction is common within marine members of the Bangiophyceae s.s. but has been rarely reported in the other classes with the exception *Rhodochaete parvula* and *Smithora naidium* (Magne, 1960; Hawkes, 1988) in the Compsopogonophyceae and *Kyliniella latvica* (Sheath, 2003) within the Stylonemato-phyceae.

The "bangiophytes" have been typically been divided into four orders: the Bangiales, Compsopogonales, Porphyridiales, and Rhodochaetales (Table 2, Gabrielson et al., 1990; Garbary and Gabrielson, 1990). In 1994, Ott and Seckbach proposed the Cyanidiales, which includes the thermophilic genera such as *Galdieria* and *Cyanidium.* The order Erythropeltidales was described earlier based on greater morphological complexity in members of the family Compsopogonaceae than in those of the Erythrotrichiaceae (Garbary et al., 1980; Silva et al., 1996). Molecular studies using plastid and nuclear genes or noncoding regions suggested that while the Bangiales and Compsopogonales were monophyletic, the Porphyridiales was polyphyletic and consisted of three clades (Ragan et al., 1994;



	Morphology	Sexual	Pit plugs	Parietal plastid	Pyrenoid	Golgi assoc.	Main habitat.
Bangiales							
Bangiaceae	multicellular	Y	Y	Y/N	Y	M/ER	marine/ freshwater
Rhodellales							
Rhodellaceae	unicellular	N	N	N	Y/N	Nu/ER	marine
Porphyridiales							
Porphyridiaceae	unicellular	N	N	N	Y/N	M/ER	soil/ marine
Stylonematales							
Stylonemataceae	multicellular/ unicellular	Y/N	N	Y	Y/N	M/ER	marine/ freshwater
Rufusiales							
Rufusiaceae	multicellular	N	N	Y	N	*	epizoic
Compsopogonales							
Compsopogonaceae	multicellular	N	Y	Y	N	ER	freshwater
Boldiaccae	multicellular	N	N	Y	N	ER	freshwater
Erythropeltidales							
Erythrotrichiaceae	multicellular	Y/N	N	N	Y	ER	marine
Rhodochaetales							
Rhodochaetaceae	multicellular	Y	Y	Y	N	ER	marine
Cyanidiales							
Cyanidiaceae	unicellular	N	N	Y	N	ER	hotsprings
Galdieriaceae	unicellular	N	N	Y	N	ER	hotsprings

Figure 1. Schematic of evolutionary relationships and characteristics among the six classes of "bangiophyte" red algae. Thicker lines represent well-supported clades from phylogenetic analyses (nuclear small subunit rRNA, unpublished data). Characteristics are based on numerous sources (Fan, 1960; Magne, 1960; Dixon, 1963; Garbary et al., 1980; Bold and Wynne, 1985; Pueschel, 1990; Broadwater and Scott, 1994). Y = yes, N = no, ER = endoplasmic reticulum, M = mitochondria, Nu = nucleus, * = unknown.

Rintoul et al., 1999; Oliveira and Bhattacharya, 2000; Müller et al., 2001a, b; Saunders and Hommersand, 2004; Yoon et al., 2006b). This observation was supported in earlier studies by Gabrielson and Garbary (1985), Gabrielson et al. (1985), and Garbary and Gabrielson (1990) using morphological, ultrastructural, and biochemical data. In addition, due to low taxon sampling, many of the molecular studies were still unable to resolve the issues of paraphyly, particularly within the order Porphyridiales (Freshwater et al., 1994; Ragan et al., 1994; Müller et al., 1998, 2001a, b; Rintoul et al., 1999; Zuccarello et al., 2000). There is tremendous potential for genomic research on the red algae to clarify taxonomic relationships and the genetic mechanisms underlying key evolutionary events (e.g., endosymbiosis). In addition, the evolution of morphological form, sexuality, and developmental processes are avenues of research that will benefit from analyses of genomic information. Currently, while genomic data within the Rhodophyta are limited, the complete nuclear, chloroplast, and mitochondrial genomes of the cyanidiophyte *Cyanidioschyzon merolae* have been sequenced and other red algal genomes will soon be available, there is a unique opportunity to extend this research into the poorly studied "bangiophyte" red algae.

Table 2. Taxonomic classification using Yoon et al., 2006b. Genera in bold have not been sequenced.

Subphlyum	Class	Order	Family	Genera
Cyanidiophytina	Cyanidiophyceae	Cyanidiales	Cyanidiaceae	*Cyanidium*
				Cyanidioschyzon
				Cyanoderma
			Galdieriaceae	*Galdieria*
Rhodophytina	Bangiophyceae	Bangiales	Bangiaceae	*Bangia*
				Dione
				Minerva
				Porphyra
				Pseudobangia
	Compsopogonophyceae	Compsopogonales	Compsopogonaceae	*Compsopogon*
				Pulvinus
			Boldiaceae	*Boldia*
		Erythropeltidales	Erythrotrichiaceae	*Chlidophyllon*
				Erythrocladia
				Erythrotrichia
				Erythrotrichopeltis
				Membranella
				Porphyropsis
				Porphyrostromium
				Pyrophyllon
				Sahlingia
				Smithora
		Rhodochaetales	Rhodochaetaceae	*Rhodochaete*
	Porphyridiophyceae	Porphyridiales	Porphyridiaceae	*Erythrolobus*
				Flintiella
				Porphyridium
	Rhodellophyceae	Rhodellales	Rhodellaceae	*Dixoniella*
				Glaucosphaera
				Neorhodella
				Rhodella
	Stylonematophyceae	Stylonematales	Stylonemataceae	*Bangiopsis*
				Chroodactylon
				Chroothece
				Colacodictyon
				Empselium
				Goniotrichiopsis
				Kneuckeria
				Kyliniella
				Neevea
				Phragmonema
				Purpureofilum
				Rhodaphanes
				Rhodosorus
				Rhodospora
				Vanhoffenia
		Rufusiales	Rufusiaceae	*Rufusia*

2. Bangiophyceae

2.1. TAXONOMY AND SYSTEMATICS

The Bangiophyceae contains the single order Bangiales and is a monophyletic class containing the genera *Bangia* and *Porphyra,* which are not monophyletic (Müller et al., 1998, 2001a, b, 2003, 2005; Broom et al., 1999, 2004; Nelson et al., 2005, 2006) as well as several newly described genera, *Pseudobangia* (Müller et al., 2005), *Dione* and *Minerva* (Nelson et al., 2005). These organisms typically have a heteromorphic life history with an alternating macroscopic gametophyte and microscopic sporophyte (conchocelis stage). The *Porphyra* gametophyte is a sheet-like thallus one to two cells thick and vary from linear (e.g., *P. linearis*) to wide and foliose (e.g., *P. umbilicalis*), while *Bangia* is a distally multiseriate filament (Sheath and Cole, 1984). The three remaining genera have filamentous morphologies, *Dione* with somewhat wide filaments (up to 150–211 μm) (Nelson et al., 2005) and *Pseudobangia* with several chloroplasts per cell (Müller et al., 2005). *Minerva* has slightly smaller cell diameters (Nelson et al., 2005).

Based on phylogenetic evidence, the sheet-like gametophytic morphology has most likely evolved multiple times within the Bangiophyceae (Müller et al., 2005; Lynch et al., 2008) and consequently the genera *Bangia* and *Porphyra* are not monophyletic. There are no known species of Bangiophyceae that are transitional between the filament and sheet morphologies. However, *Bangia maxima* is a large species (35 cm long × 6 mm in diameter) that has a distally hollow filament that is a superficially intermediate morphology between filamentous and sheet-like gametophytes. It has been proposed that there are few genetic controls on filamentous vs. sheet-like morphologies (Stiller and Waaland, 1993) and hence it is important to understand the genetic basis and the controls for the transition between the gametophytic foliose stage and the sporophytic conchocelis stage. In addition to paraphyly within this class, recent molecular studies (Müller et al., 2001a, b, 2003, 2005; Broom et al., 2004) have demonstrated cryptic diversity, which is not surprising considering its long evolutionary history.

2.2. HABITAT AND ECOLOGY

The Bangiophyceae almost exclusively consist of species attached to substrata, such as rough boulders in intertidal and upper subtidal zones in both temperate and tropical oceans (Müller et al., 1998). A single species, *Bangia atropurpurea*, occurs in freshwater habitats, such as scattered rivers and lakes in Europe and Asia, as well as the Laurentian Great Lakes of North America.

2.3. GENOMICS

The Bangiophyceae contain the economically valuable red alga *Porphyra* (a.k.a. nori, laver, sluckus), which is a 1.5 billion (US) dollar aquaculture industry

(Zemke-White and Ohno, 1999). A key watershed moment for the industry was the discovery of the full life-cycle by Kathleen Drew (1949), establishing the shell-boring *Conchocelis rosea* as the sporophyte stage of *Porphyra*. The availability of the genome for this organism will be just as significant, contributing to such areas as disease resistance, increased crop yields, and genetic engineering to increase nutritional value.

Nuclear genome sequence information within the Bangiophyceae is limited to a published EST library encompassing 3267 unique ESTs for *Porphra yezoensis* (Nikaido et al., 2000). While this EST library does not represent the full proteome of *Porphyra*, it demonstrates that more than two thirds of the ESTs showed no similarity to known genes. With the forthcoming completion of the genome of *P. umbilicalis,* the roles of many of the uncharacterized coding regions can be clarified, leading to insights about the evolution, construction, and function of these genetically divergent organisms.

Currently, there are three complete genomes for organelles of species within the Bangiophyceae (the cpDNA for *Porphyra yezoensis* and *Porphyra purpurea* and mtDNA for *P. purpurea*) and these data further add to the understanding of the genome organization and evolution of members of the class. For instance, there is clear evidence of intron transfer directly between cyanobacteria and *P. purpurea* mtDNA (Burger et al., 1999). The number of mitochondrial genes of rhodophytes are quite reduced, ~60 genes (Burger et al., 1999), indicating a substantial transfer of genetic material to the nuclear genome, a derived situation relative to the putatively more ancestral mitochondrial genomes observed in green algae, and many nonphotosynthetic eukaryotes. The mitochondrial genomes of the five sequenced rhodophytes are also highly conserved (i.e., synteny and relatively low sequence divergence), which contrasts with the high nuclear sequence divergence observed in many phylogenetic studies (Ragan et al., 1994; Müller et al., 1998, 2001a, b, 2005; Broom et al., 2004; Lynch et al., 2008). The availability of nuclear genomes would determine whether there are significantly different evolutionary pressures on the various genomes within species, accounting for the differences in divergence rates, or if the current understanding of nuclear divergence is incorrect (i.e., it is the rate of divergence accelerated for SSU rRNA in the Bangiophyceae relative to the rest of the nuclear genome or is the evolutionary rate of the mitochondrial genome slow). In addition, the genome of *P. umbilicalis* will contribute greatly to understanding evolution in the other "bangiophyte" classes.

3. Compsopogonophyceae

3.1. TAXONOMY AND SYSTEMATICS

The Class Compsopogonophyceae was proposed by Saunders and Hommersand (2004) and has received support from subsequent molecular and morphological studies (Yoon et al., 2006b; West et al., 2007a) as a well-supported monophyletic grouping. Currently, it is considered to contain three orders, the Compsopogonales,

Erythropeltidales, and the Rhodochaetales and 14 genera, including the recently described *Chlidophyllon* and *Pyrophyllon* in the Erythropeltidales (Nelson et al., 2003), and *Pulvinus* in the Compsopogonales (West et al., 2007a) (Table 2). The class is differentiated from other bangiophyte groupings by having the combination of monosporangia and spermatangia cleaved by curved walls from vegetative cells, Golgi Body-ER associations, encircling outer thylakoids in the chloroplast and a biphasic life history where known (Saunders and Hommersand, 2004) (Fig. 1). The recent report of two new members of the Erythropeltidales without the diagnostic sporangial and spermatangial development patterns (Nelson et al., 2003) was questioned by Saunders and Hommersand (2004) as to usage of this character as a synapomorphy for this particular group. The morphological types in the class ranges from prostrate discs (*Erythropeltis*, *Pulvinus*, and *Sahlingia*), to saccate or flat-bladed thalli (*Boldia*, *Chlidophyllon*, *Membranella*, *Porphyropsis, Porphyrostromium*, *Pyrophyllon* in part, and *Smithora*), to prostrate or upright filaments (rest of the genera) (Brodie and Irvine, 2003; Nelson et al., 2003).

Not all of the genera in the Compsopogonophyceae have been sequenced (Table 2) but those that fall into well-supported clades representing the orders, using both nuclear and chloroplast genes (Rintoul et al., 1999; Nelson et al., 2003; Yoon et al., 2006b). The one exception is the positioning of *Boldia* in a two-gene phylogeny as a sister group to the Compsopogonales, Erythropeltidales, and Rhodochaetales (Yoon et al., 2006b). Nonetheless, each of the orders has distinct morphological features, supporting the continued recognition of all of them as being distinct (Zuccarello et al., 2000; Yoon et al., 2006b). Since a number of the genera of this class are small epiphytes (Brodie and Irvine, 2003; West et al., 2007a), it is quite likely that there are still numerous species to be described.

3.2. HABITAT AND ECOLOGY

All the members of the Compsopogonophyceae are benthic, either being epilithic or epiphytic (Sheath and Hambrook, 1990; Brodie and Irvine, 2003; West et al., 2007a). Most are intertidal to upper subtidal, but some genera can extend into estuarine or saline inland habitats, such as *Erythrocladia setifera* (Simons et al., 2001) and *Pulvinus veneticus* (West et al., 2007a); *Boldia* and *Compsopogon* are typically freshwater in their distribution (Sheath and Hambrook, 1990). A number of species in this class are euryhaline and use low molecular weight carbohydrates to regulate their cellular osmolarity, such as floridoside (*Boldia, Compsopgon, Erythrotrichia, Rhochaete, Sahlingia,* and *Smithora*), isofloridoside (*Smithora*), or digeneaside (*Rhodochaete*) (Karsten et al., 2003).

3.3. GENOMICS

Representative members of the Compsopogonophyceae have been sequenced for various genes but largely to determine phylogenetic relationships within the group

and in relation to other bangiophytes (e.g., Rintoul et al., 1999; Zuccarello et al., 2000; Nelson et al., 2003; Yoon et al., 2006b; West et al., 2007a). Determining the genes or gene families involved in various key ecological processes, such as euryhaline tolerance or monospore recognition of a suitable substratum would be essential in understanding the evolution of this class.

4. Cyanidiophyceae

4.1. TAXONOMY AND SYSTEMATICS

The cyanidiophytes are a monophyletic lineage sister to other red algae (collectively the Rhodophytina [Yoon et al., 2006b] or Rhodophyta [Saunders and Hommersand, 2004]) and have recently been elevated variously to the taxonomic rank of subphylum (Cyanidiophytina [Yoon et al., 2006b]) and phylum (Cyanidiophyta [Seckbach, 1987; Doweld, 2001; Saunders and Hommersand, 2004]). This group contains the single class, Cyanidiophyceae. Members of the group are unicellular, either being spherical or oblong in shape +/– thick cell walls and containing a single plastid, one to three mitochondria, a nucleus, a vacuole, and floridean starch (Fig. 1). Such simple morphologies have influenced taxonomy in the group and results in paraphyletic relationships among the taxa.

Phylogenetic evidence supports the hypothesis that the Cyanidiophyceae is an early diverged lineage of red algae, most likely greater than 1.3 BYA (Müller et al., 2001a, b; Yoon et al., 2006b). Despite the age of the lineage, there is currently little recognized species diversity and it is unclear whether the low diversity is due to the presence of cryptic species (morphologically indistinguishable) or if the selective pressures in highly specialized environments and asexuality have maintained species integrity. Recent studies (Ciniglia et al., 2004; Toplin et al., 2008) indicate support for cryptic species.

There are at least four distinct lineages in the Cyanidiophyceae (Ciniglia et al., 2004; Pinto et al., 2007), including the *C. merolae*/*Galdieria maxima* lineage, the *Galdieria* spp. lineage (excluding *G. maxima*), the *Cyanidium caldarium* lineage, and the mesophylic *Cyanidium* spp. lineage. Within these lineages, there are currently three recognized genera (*Cyanidium, Cyanidioschyzon,* and *Galdieria*) and seven species (Yoon et al., 2006a; Pinto et al., 2007). Owing to their simple morphologies, the relationship between morphological similarity and genetic divergence is not clear-cut in the Cyanidiophyceae, and the circular or oblong unicellular morphologies are most likely convergent characters. For example, in a recent study (Toplin et al., 2008), an isolate with the same *Galdieria*-type morphology (cell walls and spherical shape) was nearly identical to *C. merolae* (lack of cell wall and oblong shape) over the *rbc*L and nuclear SSU rRNA gene loci. Increased sampling of environmental isolates is essential for clarity in the evolutionary relationships and species diversity within this class.

4.2. HABITAT AND ECOLOGY

The Cyanidiophyceae are thermophilic (50–55°C) and acidophilic (pH 0.5–3.0) and thrive in extreme environments such as hot springs, sulfur fumes, and within rocks (endolithic) (Fig. 1, Ciniglia et al., 2004; Toplin et al., 2008). The endolithic species are photosynthetic in the upper layers and the remaining individuals exist heterotrophically because of light attenuation. Due to their obligate extremophile habit, species within this class exhibit a disconnected biogeography. Consequently, there was a strong likelihood of higher species diversity than currently recognized. This possibility is consistent with the observed molecular phylogenetic patterns of cyanidiophyceaen isolates from New Zealand, Japan, and Yellowstone National Park (Toplin et al., 2008). There also appear to be ecotypes within species or lineages of the Cyanidiophyceae. For example, *Galdieria sulphuraria* was observed in both dry endolithic and sulfur fume-mediated humid environments, a split mirrored in the distinct evolutionary histories and geographic distributions of the two groups (Ciniglia et al., 2004). This observation led to the recognition of the endolithic lineage as a new species, *Galdieria phlegrea* (Pinto et al., 2007). Moreover, *Galdieria* species are very versatile in habitat (e.g., tolerant to desiccation) and trophic potential (e.g., both heterotrophic and mixotrophic) than other groups within this class.

4.3. GENOMICS

Owing to the small size, genome reduction and morphological simplicity of these organisms, more genomics work has been done within the Cyanidiophyceae than any other red algal class. Nonetheless, only a single red algal nuclear genome has been completely sequenced, the primitive *C. merolae* 10D (Matsuzaki et al., 2004). This species has one of the smallest genomes among photosynthetic eukaryotes with 16.5 Mbp organized into 20 chromosomes containing 5,331 genes, the majority (86.3%) of which are expressed (Matsuzaki et al., 2004). In addition, this genome has very different features relative to the only other cyanidiophyte genome with appreciable data available including a considerable difference in G+C content (*C. merolae:* 55%; *G. sulphuraria* 37%) and the presence of introns (*G. sulphuraria* contain short, 45–60 bp introns) (Barbier et al., 2005).

Comparative genomics of members of this class can establish what genes are under selective pressures, an important step in understanding gene–ecosystem relationships. Much can be derived from comparing the genomes of *G. sulphuraria* and *C. merolae*. These two species diverged early in the evolution of the Rhodophyta (Yoon et al., 2006b) and show many cellular and biochemical differences (Weber et al., 2007). Based on preliminary genome sequencing results, utilizing approximately 70% of the completed genome of *G. sulphuraria,* roughly 30% of its genes did not have orthologs in *C. merolae* (Barbier et al., 2005). Some of the genes observed in *Galdieria* without orthologs in *Cyanidioschyzon* included membrane transporters that facilitate the acquisition of carbohydrate substrates. This finding

is consistent with the ability of *G. sulphuraria* to grow in different trophic states and utilize up to 50 different types of carbohydrates.

Currently, two cyanidiophyte chloroplast genomes (*C. merolae* strain 10D and *C. caldarium*) and one cyanidiophyte mitochondrial genome (*C. merolae*) have been sequenced. The chloroplast genome of *Cyanidium caldarium* contains a cluster of genes that play a role in thermotolerance and stress endurance (Glockner et al., 2000). Additionally, a large number of genes (19), 8.2% of the total gene complement of the chloroplast, were ancestral and maintained most likely due to the extreme living conditions of *Cyanidium caldarium* (Glockner et al., 2000). Other eukaryote extremophiles, including some diatoms and green algae, have different strategies for dealing with extreme environments, including formation of calcified cells and thick cell walls. Hence, there is significant potential for these organisms in biotechnology (e.g., the ability of these organisms to deal with acidic environments and heavy metals).

5. Porphyridiophyceae

5.1. TAXONOMY AND SYSTEMATICS

Resolving the taxonomic status of the order Porphyridiales has been one of the most difficult issues within bangiophyte taxonomy, as this order has long been recognized as a polyphyletic entity based on morphology, ultrastructure, and molecular phylogenies (Fritsch, 1945; Garbary et al., 1980; Gabrielson et al., 1985; Garbary and Gabrielson, 1990; Müller et al., 2001a, b; Yoon et al., 2006b). Garbary and Gabrielson (1990) noted that this order was an assemblage based on a unicellular to palmelloid morphology. Traditionally, the Porphyridiales was separated into two families, the Porphyridiaceae and Phragmonemataceae, with the first family being composed of unicellular and multicellular taxa with single stellate plastids and pyrenoids (Fig. 1, Garbary et al., 1980; Gabrielson et al., 1990) while taxa within the Phragmonemataceae do not have stellate plastids or pyrenoids (Fig. 1, Garbary et al., 1980; Gabrielson et al., 1990). Recent molecular phylogenies (Müller et al., 2001a, b; Yoon et al., 2006b) have clearly identified three lineages that comprise what was previously known as the order Porphyridiales. Using multiple gene phylogenies, Yoon et al. (2006b) divided this order into three separate classes: the Rhodellophyceae, Stylonematophyceae, and the Porphyridiophyceae. The Porphyridiophyceae thereby contains three unicellular genera (Table 2): *Porphyridium, Erythrolobus*, and *Flintiella.* Unicellular morphology in the red algae has been suggested to be indicative of a primitive nature; however, it is possible that multicellularity is secondarily lost (Garbary et al., 1980). The three genera all have ER–mitochondria–Golgi Body associations, but lack peripheral, encircling thylakoids (Fig. 1; Scott et al., 1992, 2006); however, in molecular phylogenies there are three distinct clades (Yoon et al., 2006b).

5.2. HABITAT AND ECOLOGY

Members of the genus *Porphyridium* are typically observed on damp and sometimes acidic soils in forests and greenhouses (Ott, 1987; Sheath and Sherwood, 2002). *Porphyridium sordidum* forms olive-green mats on soils, whereas *P. purpureum* is observed to form a reddish layer on damp surfaces and soils though both species tend to co-occur (Ott, 1987). *Erythrolobus* was isolated from submerged stones on South Padre Island, Texas, in the Gulf of Mexico (Scott et al., 2006).

5.3. GENOMICS

The unicellular habit of this class raises interesting questions regarding the evolution of or loss of multicellularity within the red algae. Advances in genomics, in particular the discovery of genes that play a role in multicellular development, will aid in determining if functional genes are still present within the genomes of these unicellular taxa and address the hypothesis of whether or not this characteristic is primitive. In addition, high-efficiency genetic transformation has been documented in *Porphyridium* (Lapidot et al., 2002) and raises the possibility of future expansion of transformation in other red algae including those that are of economic interest (e.g., *Porphyra*).

6. Rhodellophyceae

6.1. TAXONOMY AND SYSTEMATICS

The Rhodellophyceae was established by Cavalier-Smith (1998) and was supported by Saunders and Hommersand (2004); however, the latter study included two clades of Porphyridiales and the Stylonematales but did argue that this classification was an interim solution and that this group does not appear to be monophyletic. Hence, the taxonomic scheme suggested by Saunders and Hommersand (2004) still resulted in paraphyly within the Rhodellophyceae. More recently, Yoon et al., (2006b) separated the Rhodellophyceae and proposed three monophyletic classes: Rhodellophyceae, Porphyridiophyceae, and Stylonematophyceae, as well as a new order and family, Rhodellales and Rhodellaceae, within the Rhodellophyceae (Table 2). This proposal is supported by ultrastructural analyses of the four genera (Fig. 1), including the newly proposed *Neorhodella* (Patrone et al., 1991; Scott et al., 1992, 2008; Broadwater et al., 1995). All these genera have chloroplasts with several lobes and plastoglobuli clusters plus Golgi cisternae appressions in recently divided and young cells (Scott et al., 2008). In addition, *Dixoniella*, *Neorhodella*, and *Rhodella* accumulate mannitol as the low molecular weight carbohydrate osmolyte (Karsten et al., 2003; Scott et al., 2008); the status of *Glaucospheara* in this regard is currently not known.

In phylogenetic analyses of members of the Rhodellophyceae, the genera are well separated (Müller et al., 2001b; Yokoyama et al., 2004). However, a problematic taxon is *Dixoniella grisea,* which is typically positioned on extremely long branches within molecular phylogenies sister to *Rhodella* and *Glaucosphaera* (Müller et al., 2001a, b; Yokoyama et al., 2004; Yoon et al., 2006b). In fact, Yokoyama et al. (2004) noted that *Dixoniella* groups with *Glaucosphaera* in 18S rDNA phylogenies and this clade is sister to *Rhodella*. This finding is similar to that noted by Yoon et al. (2006b) in their combined plastid protein sequence analyses. *Glaucosphaera* is the one member of this class that does not contain a pyrenoid but Yokoyama et al. (2004) concluded that this was an autapomorphy. *Neorhodella* is considered to have multiple pyrenoid-like regions on the inner tips of the chloroplast lobes (Scott et al., 2008). All of the genera in this class are unicellular and hence it is very likely that there is greater diversity than currently recognized.

6.2. HABITAT AND ECOLOGY

The four genera of the Rhodellophyceae have been mostly studied from cultured material and ecological data are limited. *Neorhodella* and *Rhodella* have been largely isolated from marine or estuarine environments (Karsten et al., 2003; Scott et al., 2008). *D. grisea* has also been collected in inland thermal basins and estuarine habitats and is euryhaline (Pekárková et al., 1988; Scott et al., 1992). *Glaucosphaera* can be grown on freshwater or diluted seawater media, also indicating that it is adapted to a wide range of salinities (Broadwater et al., 1995; Karsten et al., 2003).

6.3. GENOMICS

Microalgae have become a matter of considerable interest in terms of biotechnological applications and the members of the Rhodellophyceae are potential candidates in their unique storage of mannitol. Comparison of future EST libraries of members of this class with sequenced and prospective red algal genomes will highlight evolutionary differences and genes important in the synthetic pathways of these compounds.

7. Stylonematophyceae

7.1. TAXONOMY AND SYSTEMATICS

The class Stylonematophyceae was proposed by Yoon et al. (2006b) and encompasses one of the three clades formerly within the Porphyridiales s.l. or Rhodellophyceae, the latter of which was postulated by Saunders and Hommersand (2004). However, as noted previously, both these clades were clearly polyphyletic

within molecular phylogenies, which in turn warranted separation into three separate clades or classes (Müller et al., 2001a, b; Yoon et al., 2006b). Most taxa within this class typically have a single stellate plastid with a pyrenoid (Fig. 1); sexuality is rare and has only been reported in the freshwater genus *Kyliniella* (Sheath, 2003). Morphology ranges from unicells (*Rhodosorus* and *Rhodospora*) to pseudo-filamentous forms (e.g., *Chroodactylon* (Fig. 1)). Taxa typically contain digeneaside and/or sorbitol as a low molecular weight carbohydrate with the exception of *Rufusia*, which contains floridoside (Karsten et al., 2003; West et al., 2007b); however, this property has not been determined in all taxa within this class. Currently, there are 15 known genera within this class and six of these have not been sequenced for molecular gene phylogenies. The class, however, is a well-supported monophyletic group and contains two orders, the Stylonematales and Rufusiales, the latter of which was described recently and contains the monotypic genus *Rufusia* (Zuccarello et al., 2008).

7.2. HABITAT AND ECOLOGY

Taxa within the Stylonematophyceae are generally rare and are often overlooked in floras, as they typically occur as epiphytes on larger algae or macrophytes; thus, it is quite likely that numerous new species or genera have yet to be described within this class. For example, *Chroodactylon ornatum* is a common epiphyte on *Cladophora* in the Laurentian Great Lakes (Vis and Sheath, 1993; Sheath, 2003); however, it is typically not observed unless one is specifically searching for it. In an analysis of 1,000 streams, Vis and Sheath (1993) noted the presence of *Chroodactylon* in seven streams, all epiphytic on either *Cladophora* or *Rhizoclonium*. This genus is also marine in its distribution like many of the other members of the class. For example, *Bangiopsis* is typically observed attached to rocks in the intertidal zone. One unusual species, *Rufusia pilicola*, is epizoic and grows in the furrows of hairs in two- and three-toed sloths from Panama and Costa Rica (Wujek and Timpano, 1986).

7.3. GENOMICS

This class is interesting from an evolutionary perspective and, considering a parsimonious explanation for loss of multicellularity within this clade, it is very likely that the unicellular habit is derived. In addition, the enigmatic genus *Rufusia* raises questions regarding the evolution and adaptations to its unusual habitat.

8. Summary

We are now poised for a revolution within red algal biology with several major genome projects underway, including *P. umbilicalis* (Bangiophyceae). These projects are key to advance comparative research on mechanisms governing development

and sexual reproduction, endosymbioses, morphological form in the red algae and particularly in the six classes discussed in this chapter. As noted previously, morphological form, life-history, habit, and other characteristics are highly divergent among the "bangiophytes" and we are just beginning to address these complexities and attempt to achieve taxonomy that reflects the evolutionary history. As was so nicely stated by Grossman (2005), "a strong knowledge base with respect to biological and molecular aspects of an organism will have a major impact on the exploitation of genomic information." This statement is certainly true for the classes presented here in the interpretation of future genomic information.

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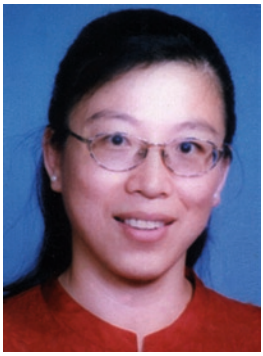

GENOMIC CONTRIBUTIONS TO UNDERSTANDING THE EVOLUTION OF RED ALGAL PLASTIDS AND PIGMENT BIOSYNTHESIS

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1. Introduction

Red algae, along with cyanobacteria, perform oxygenic photosynthesis using a combination of chlorophyll and accessory light-harvesting pigments, known collectively as phycobiliproteins. These pigments are structurally arranged with linker polypeptides on the outer thylakoid membrane and transfer light energy to chlorophyll *a* of photosystem II within the thylakoid. The bilin proteins include phycocyanin, allophycocyanin, and phycoerythrin and, when combined with the linkers, form phycobilisomes. Linker polypeptides are usually nonpigmented and function to direct photon energy to the core of phycobiliproteins and then chlorophyll.

These cellular components in red algae represent an evolutionary intermediate between the prokaryotic and higher eukaryotic photosynthetic machineries that have resulted in the oxygenation of Earth. Critical to this evolutionary process has been the endosymbiotic events that gave rise to plastids and their origins and distribution throughout phylogeny. An interesting aspect of red algae photosynthesis and the phycobiliproteins, in particular, is the colocalization of the encoding genes in the chloroplast DNA while certain linker polypeptides are on the nuclear genome.

This chapter will introduce current information from genetic and genomic studies on red algal plastid evolution, with particular reference to the molecular biology of photosynthetic pigment biosynthesis. The phylogeny of the plastid encoded light harvesting complex genes and their regulation will also be shown to reflect the ecophysiology of red algae and their relatives.

2. Phylogeny of Red Algal Plastids

It has long been clear that the red algal plastid, unlike that of most protist groups, is a "primary" plastid, a direct descendent of a symbiotic event between a cyanobacterium and a eukaryote. A crucial clue that this plastid is indeed primary

is the presence of two bounding membranes, rather than three or four membranes, indicative of further symbiotic events, commonly present in other protists.

Until recently, what remained unclear was whether the same endosymbiotic event that led to red algae also gave rise to the Viridiplantae (green algae and land plants) and the Glaucophyta, or whether several separate events led to each plastid type (Bhattacharya and Medlin, 1995; McFadden, 2001). Major differences exist between the plastid types. For example, green plant plastids contain chlorophylls *a* and *b* and stacked thylakoids, while red algae and glaucophytes contain chlorophyll *a*, unstacked thylakoids, and phycobilisomes. Research over the past 10 years is now indicating that despite these differences, all plastids probably had a single common ancestry.

2.1. EVIDENCE FOR A SINGLE PRIMARY ENDOSYMBIOTIC EVENT GIVING RISE TO ALL PLASTIDS

There are several lines of evidence that all plastids evolved in a single evolutionary event.

1. Gene order and gene content in complete red algal genomes: The monophyly of plastids is supported by common gene content in all plastid genomes, the presence of plastid-specific gene clusters that are distinct from those in cyanobacteria, and the conservation of the plastid-protein import machinery and protein-targeting signals (Stoebe and Kowallik, 1999, Matsuzaki et al., 2004, McFadden and van Dooren, 2004). Six complete red algal plastid genomes have been sequenced from the species *Cyanidioschyzon merolae, Porphyra yezoensis, Gracilaria tenuistipitata* var. *liui, Porphyra purpurea, Cyanidium caldarium*, and *Galdieria sulphuraria*. The plastids of these species contain a more complete set of plastid genes than is known from other photosynthetic eukaryotes (Reith and Munholland, 1993; Glöckner et al., 2000; Matsuzaki et al., 2004; Weber et al., 2004; Hagopian et al., 2004; Barbier et al., 2005). For example, *Porphyra* has 70–80 additional genes when compared to average higher plant plastid genomes (Reith and Munholland, 1993, 1995). The *Porphyra* and *Gracilaria* genomes contain cyanobacterial-like features, including a complete set of tRNAs, more genes encoding transcriptional regulators of a large number of genes, more operon-like structures similar to the ones found in cyanobacteria and the absence of introns. *Gracilaria* additionally contains the cyanobacterial-like features of a single base overlap of atpF and D and the presence of the *psa*A–*psa*B–*rps*14 operon. The mosaic pattern of Calvin cycle enzymes in *Cyanidioschyzon merolae* is the same as that in Viridiplantae (Matsuzaki et al., 2004). As many of the original endosymbiont genes have been laterally transferred to the nucleus, the products of these genes must be transported back to the plastid in order for it to function. The transit peptides that direct the plastid proteins from the nucleus back to the plastid are very similar in all plastid-containing organisms,

including Viridiplantae and red algae (Lang et al., 1998, McFadden and van Dooren, 2004). All plastids also contain one peptide, Tic110, that is only present in eukaryotic plastids and is absent in cyanobacteria, indicating that it is a shared derived character (McFadden and van Dooren, 2004). Additionally, a family of genes present in red algae that are involved in plastid solute transport appear to have originated in the Plantae ancestor (Weber et al., 2006).

2. Phylogenetic analyses of nuclear and mitochondrial genes: Initial phylogenetic analyses based on the nuclear genes small subunit ribosomal RNA (Bhattacharya and Medlin, 1995; Van de Peer and De Wachter, 1997) and DNA-dependent RNA polymerase II (RPB1) sequences indicated that it was very likely that red algae and Viridiplantae had originated in two events (Stiller and Hall, 1997; Stiller et al., 2001). However, phylogenetic analysis of the nuclear elongation factor 2 (EF-2) gene (Moreira et al., 2000) and mitochondrial genes supported the monophyly of red algae and Viridiplantae (Burger et al., 1999). Increasingly, concatenated alignments of multiple nuclear genes have been used in phylogenetic analyses. Analyses of four to fifty genes, including more robust outgroups, species of Glaucophyta and Cyanobacteria and more slowly evolving red algal and Viridiplantae taxa, have strongly supported a single origin of plastids (Nozaki et al., 2003; Rodríguez-Ezpeleta et al., 2005). A phylogenetic analysis of conserved nuclear-encoded plastid-targeted proteins of cyanobacterial origin found strong support for the early divergence of glaucophytes as a sister group to the red/green lineage (Reyes-Prieto and Bhattacharya, 2007). The results of the early analyses of nuclear genes may have been affected by a lack of available molecular sequences and of tree reconstruction artifacts, such as long branch attraction, that unequally affect different genes, particularly when outgroups are distant (Moreira and Philippe, 2001). Rodríguez-Ezpeleta et al. (2005) found that an analysis of 30,000 amino acid positions was necessary to robustly recover the phylogenetic signal.

2.2. WHAT WAS LOST IN THE RED ALGAL PLASTID LINEAGE AND WHEN DID IT SPLIT OFF?

Glaucophyte plastids and cyanobacteria share some common characters, which were lost in the common ancestor of red and green algae: the presence of a peptidoglycan deposition between the two organelle membranes and carboxysomes (Reyes-Prieto and Bhattacharya, 2007). In the red algal and Glaucophyte plastid lineage, chlorophyll *b* was additionally lost.

Molecular clock analysis using an alignment of six plastid genes, calibrated by fossil evidence, suggest that the primary endosymbiosis may have occurred around 1.6 billion years ago (Yoon et al., 2004). This estimate has been supported by phylogenetic analyses of nuclear genes, which suggest that primary plastids originated 1.6–1.5 billion years ago (Hedges et al., 2004). The split of the red and green algal lineages appears to have occurred about 1.5 billion years ago (Yoon et al., 2004).

3. The Biosynthesis of Red Algae Pigments

The major accessory pigments in red algae are the phycobilins, which are responsible for their red color, as indicated by its name. There are three types of phycobilin pigments: Phycocyanobilin, which has an absorbance spectrum of 590–643 nm, phycoerythrobilin (550–568 nm), and phycourobilin (495 nm) (Toole and Allnutt, 2003). Bilin synthesis in red algae has been proposed to follow the pathway as shown in Fig. 1 (Rhie and Beale, 1994, Frankenberg et al., 2001):

Phycobiliproteins are the major antenna system in red algae, where they are organized into supramolecular complex of phycobilisomes. All phycobiliprotein encoding genes, *Cpc*s, *Apc*s, and *Cpe*s, are located in the chloroplast genome and are closely related to cyanobacterial phycobiliproteins.

The first step of phycobilin formation is the conversion of protoheme to biliverdin IX alpha (Fig. 1), catalyzed by heme oxygenase (encoded by gene *pbsA*). In red algae, this gene is mostly located in the plastid genome and demonstrates strong homology to genes in other algae (plastid genes) and cyanobacteria (Toole and Allnutt, 2003). The recently released complete genomic sequence of the red alga *Cyanidioschyzon merolae (C. merolae*) (Matsuzaki et al., 2004) showed that it possessed a unique heme oxygenase, which is encoded by the *PbsA* gene, located in the nucleus. Such nuclear *pbsA* genes have also been found in a unicellular red alga, *Cyanidium caldarium* (Glöckner et al., 2000; Nozaki et al., 2007). A phylogenetic comparison reveals that heme oxygenases in *C. caldarium* and *C. merolae* are different from the heme oxygenases of plastid origin in the multicellular red alga *Porphyra* (Fig. 2). The differences suggest that the secondary endosymbiosis in the red lineage (e.g., *Guillardia theta*) (Lopez-Garcia and Moreira, 1999) might have taken place after the separation of the ancestors of red algae (unicellular red algae) and the higher red algae. A phylogenetic analysis that we have conducted of heme oxygenase (Fig. 2) indicates that heme oxygenases in unicellular algae (red and green lineages) are more closely related to higher plant heme oxygenases, and the heme oxygenases in animals and humans are remotely related to the genes found in cyanobacteria.

The final steps for producing phycobilins (phycoerythrobilin and phycocyanobilin) are catalyzed by ferredoxin-dependent bilin reductase with different double-bond specificities (Fig. 1). This gene family codes for bilin reductase of several different but closely related proteins, which include phycocyanobilin:ferredoxin oxidoreductase (*PcyA*),15,16-dihydrobiliverdin:ferredoxin oxidoreductase (*PebA*),

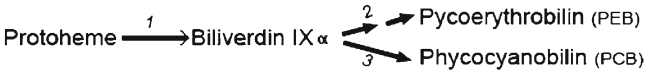

Figure 1. Biosynthesis of Bilins. Enzyme 1 is heme oxygenase (encoded by *PbsA*); enzyme 2 represents 1 5,16-dihydrobiliverdin:ferredoxin oxidoreductase EC:1.3.7.2 (*PebA*) and phycoerythrobilin:ferredoxin oxidoreductase EC:1.3.7.3 (*PebB*), and enzyme 3 is phycocyanobilin:ferredoxin oxidoreductase EC:1.3.7.5 (*PcyA*).

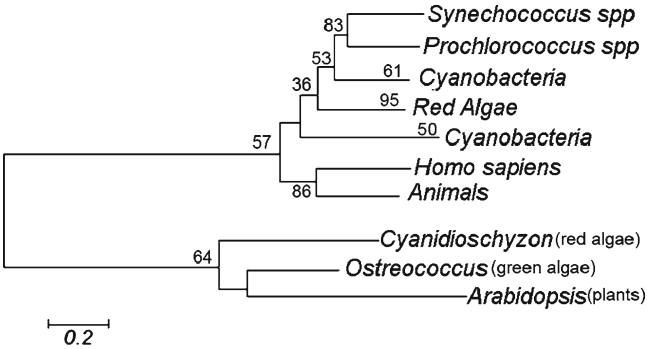

Figure 2. A simplified phylogenetic tree of heme oxygenases (HO), inferred using the Neighbor Joining algorithm. Each terminal group represents several genomic sequences of a particular lineage. Five species of *Synechococcus* spp. and *Prochlorococcus* spp., respectively, are included. The 16 HO sequences of cyanobacteria are clustered into two paraphyletic groups. Six selected animal and two *Homo sapiens* HO sequences are included. The *Arabidopsis* group represents higher plants and includes four HO sequences. The red algae group includes the sequences of *R. violacea* (AAB66516), *P. purpurea* (NP_053881), *G. tenuistipitata var. liui* (AAT79699), *P. yezoensis* (YP_536953), *G. theta* (NP_050754), *R. salina* (YP_001293565). The unicellular red algae *Cyanidioschyzon* is shown to form a different group. The phylogenetic tree is derived from an alignment of full-length heme oxygenase amino acid sequences (using ClustalW) excluding the N/C terminals and the gap regions. The bootstraps were calculated using 1,000 replicates and are stated at the branch nodes. The numbers on the end of branches represent the group bootstrap support values.

and phycoerythrobilin:ferredoxin oxidoreductase (*PebB*). However, the location of those genes in red algae is not yet clear, as the genomic data are limited. In the unicellular red alga *C. merolae*, there is a single copy of *PcyA*, which is located on the 7th chromosome and no homologies for *PebA*/*B* are found. The absence of *CpeA*/*B* and *PebA*/*B* in *C. merolae* indicates that there is no phycoerythrin in *C. merolae*. The complete chloroplast genome of *C. caldarium* reveals the absence of *CpeA* and *CpeB* genes as well. Although no nuclear DNA information is available for this organism, we propose that there is a simpler structure of phycobiliproteins in unicellular red algae when compared with the classical supercomplexes of phycobilisomes in multicellular red algae. The phycoerythrins may be a later evolved bilin, since the classical $(\alpha\beta)_6$ structure of phycoerythrins have been discovered and characterized in a variety of multicellular red algae (Apt et al., 1993). Thomas and Passaquet (1999) reported the properties of a novel phycoerythrin protein forming in a unicellular red alga, *Rhodella reticulata* strain R6. They proposed that the apoprotein of the β-subunit of the *R. reticulata* R6 phycoerythrin in $\alpha\beta_6$ structure is specified by a monocistronic *RpeB* chloroplast gene that is split into three exons.

The updated genome sequences provide the genomic information for understanding the chlorophyll biosynthesis pathway. Chlorophyll *a* is the only type of chlorophyll found in red algae. The complete genome of *C. merolae* indicates that all the proteins involved in Chl *a* biosynthesis are present, but they are distributed

in several different chromosomes. Genes for only two enzymes, Magnesium-protoporphyrin IX cyclase (*AcsF*) and Magnesium-chelatase subunit I (*ChlI*), are located in the chloroplast genomes (see Table 1).

There are no light-independent protochlorophyllide reductases found in the *C. merolae* genome. However, most algae and cyanobacteria have the ability to synthesize chlorophylls in the absence of light (Beale, 1996). Three subunits *ChlL*, *N*, and *B* are required for light-independent protochlorophyllide reductase, i.e., to catalyze the reaction to produce chlorophylls in the dark. Those three genes are found in *Porphyra* chloroplast genome, but not in *C. merolae*.

ChlI, D, and H are three subunits of the magnesium-chelatase enzyme that inserts Mg into Protoporphyrin IX (5). The genes of plastid-encoded *ChlI* and nuclear-encoded *ChlD* are related (Jensen et al., 1996). Li et al. (2006) showed that *ChlI* genes are monophyletic and indicated that red algal *ChlI* may have originated from a cyanobacterial endosymbiont. This gene is plastid encoded in all known plants and algae. On the other hand, the evolutionary relationship of *ChlD* indicates a parallel relationship between the red algal lineage and other algal groups, as it is a nuclear-DNA encoded protein (Li et al., 2006).

The final step of chlorophyll synthesis is catalyzed by chlorophyll *a* synthase (encoded by *ChlG* gene). This enzyme is widespread in all photosynthetic organisms. The results of Blast searches provide strong support for a monophyletic origin of plastid-encoded proteins. Interestingly, the apparently mosaic origin of chlorophyll synthesis genes in red algae is similar to that found for heme biosynthesis in eukaryotes (Oborník and Green, 2005). In all photosynthetic eukaryotes, the heme biosynthesis pathway has a mosaic evolution. These enzymes involved have been shown to have either cyanobacterial (plastid), proteobacterial (mitochondrial), and cytosolic (eukaryotic nucleus) origins. Interestingly, the ferrochelatase of red algae (*Porphyra yezoensis, Cyanidioschyzon merolae,* and *Galdieria sulphuraria*) appears to have originated from the apicomplexan-proteobacteria lineage, as opposed to the expected diatom-plastid-cyanobacteria branch (Oborník

Table 1. The distribution of Chl synthesis genes in *C. merolae*.

Enzymes	Genes	Location
Magnesium-protoporphyrin IX cyclase	*AcsF*	Chloroplast
Magnesium chelatase	*Chl I*	Chloroplast
	Chl D	C13
	Chl H	C02/C15
Mg-protoporphyrin *O*-methyltransferase	*Chl M*	C09
Chl *a* synthesase	*Chl G*	C20
Light-dependent Protochlorophyllide reductase	*POR*	C07/C14
Heme oxygenase	*PbsA*	C08
Phycocyanobilin:ferredoxin oxidoreductase	*PcyA*	C07
Phycoerythrobilin:ferredoxin oxidoreductase	*PebA*/*B*	NO

C, chromosome; number after C represents the number of chromosomes.

and Green, 2005). This enzyme catalyzes the first committed step in heme and bilin synthesis and, as in *Plasmodium*, may be a proteobacterial replacement of the original cyanobacterial-plastid type as found in diatoms.

4. Localization and Regulation of Phycobiliprotein Production

The chromophores, or bilins, of the phycobiliproteins are termed phycocyanobilin, phycoerythrobilin and phycourobilin. In rhodophytes, these proteins are encoded by genes in the plastid genome and their expression is coordinately regulated. The involvement of nuclear-encoded linker peptides is also coordinated with those of the plastid. Light quality and quantity is the main regulator of phycobiliprotein gene transcription and accumulation in the chloroplast (Reith, 1995). However, heme also functions to regulate the specific expression of bilins, as opposed to other structural components of photosynthesis in red algae (Troxler et al., 1989). Low light conditions tend to induce the accumulation of phycobiliproteins and their transcripts, as well as increasing the length of their chromophore rods. An interesting aspect of the regulation of photosynthetic pigments in red algae and cyanobacteria is the chromatic adaptation in response to exposure to varying light quality. Here, for example, cells grown in green light will show increased transcription and accumulation of the phycoerythrin structural subunits. Phycocyanin genes have similar response to a red light source (Grossman et al., 1995, Wollman, 2001, Stowe-Evans and Kehoe, 2004, Kehoe and Gutu, 2006).

The biliprotein content of red algae has also been shown to be adapted to particular niche environments as well being resistant to environmental fluxes. For example, the phycocyanin of the acido- and thermophilic *Cyanidium caldarium* is functional between 10°C and 50°C (Eisele et al., 2000). *Phyllophora anarctica*, which is found under ice in the Antarctica, has similarly adapted its phycobilisome to contain a phycourobilin-abundant phycoerythrin, thus affording the harvesting of blue wavelength light (MacColl et al., 1996)

Alternatively, the lack of other nutrients results in the degradation of phycobilisomes. This is most notable under nitrogen-depleted growth where increased protease activity against the phycobilisomes has been shown. Iron limitation results in reduced bilin and Chl *a* biosynthesis, as well as the formation of a poorly defined alternate chlorophyll–protein complex (Reithman et al., 1988; Desquilbet et al., 2003). In cyanobacteria, the depletion of iron results in upregulation of *PsbC* homologues formation (Laudenbach and Strauss, 1988) and is proposed to result in the formation of a stored form of chlorophyll, as a pigment–protein complex, which could become active when iron is replete. Also in cyanobacteria, and probably red algae, sulfur depletion results in the cancellation of phycocyanin production.

Functionality of the phycobilisome is dependent on linker polypeptides that are encoded by plastid DNA in red algae. Little is known regarding the phylogeny and regulation of biosynthesis of the linker peptides. The chromophorylated gamma subunit of phycoerythrin acts as a linker but is nuclear encoded and is

transported to the chloroplast to stabilize phycoerythrin hexamers (Apt et al., 1993). Due to the presence of phycoerythrobilin and particularly phycourobilin in the gamma subunit, the phycobilisomes of red algae are able to absorb shorter wavelengths (ca. 500 nm) of light energy. The regulation of gene transcription in algal plastids and green plants is generally under the control of nuclear-encoded factors; however, rhodophytes possess transcriptional sigma factors related to those of cyanobacteria encoded within their plastid genome and interact with the plastid RNA polymerases (Oikawa et al., 1998).

A functional analysis of the *Cyanidioschyzon merolae* plastid genome using a microarray has shown that the transition from dark to light and from low light to light differentially regulates four plastid transcription factors (Minoda et al., 2005). These proteins have a bacterial ancestry and work in concert with nuclear-encoded sigma factors. It has also been observed in *Arabidopsis thalliana* that one of the nuclear-encoded plastid factors (SIG2), in turn, regulates tetrapyrrole biosynthesis, the first step in bilin production (Nagashima et al., 2004). Similarly, the plastid genome of *Porphyra purpurea* contains genes for the regulating transcription and biosynthesis of pigments (Reith, 1995).

5. Summary

The plastids of red algae represent a unique lineage and are highly divergent, both from those of oxygenic phototrophic prokaryotes and the well-known and well-studied plastids of Viridiplantae, the higher plants. In this era of global climate change, there is a strong need to understand photosynthetic processes and their genetic and regulatory basis, particularly as they relate to biosequestration and potential biofuel development. As such, red algal plastids are a critical model for the understanding of photosynthesis and its associated molecular structures. To date, however, very few red algal taxa have been the subject of modern "omics"-based research. As of 2008, complete genomes are available for only two red algal taxa (*C. merolae* and *G. sulphuraria*); however, one more is in process, and several plastid genomes are available. Considering the diversity present in this group, there is a critical need for resourcing the analysis of more red algal genomes. The opportunity to further our knowledge regarding the origin and distribution of plastids is greatly aided by the ability to sequence entire genomes. Similarly, the sequencing of genomes and EST libraries, together with the use of microarray and proteome analyses, provides a system for the characterization of photosynthetic pigments and their trafficking between nucleus, cytoplasm, and the chloroplast.

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HOW HAVE GENOME STUDIES IMPROVED OUR UNDERSTANDING OF ORGANELLE EVOLUTION AND METABOLISM IN RED ALGAE?

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1. Introduction

The genomic era has not left out red algae. There is now a complete genome of the cyanidiophycean *Cyanidioschyzon merolae* (Matsuzaki et al., 2004; Barbier et al., 2005; Misumi et al., 2005), a nearly complete genome of the cyanidiophycean *Galdieria sulphuraria* (Barbier et al., 2005), and genome sequencing projects underway for the bangiophycean *Porphyra* sp. and the florideophyceae *Chondrus crispus.* In addition to these complete genome sequences, a number of expressed sequence tags (EST) studies with a range of objectives on red algae such as the bangiophyceans *Porphyra haitanensis* (Xiaolei et al., 2007) and *Porphyra yezoensis* (Nikaido et al., 2000; Asamizu et al., 2003; Kitade et al., 2008), and the florideophyceae *Chondrus crispus* (Collén et al., 2006), *Gracilaria changii* (Teo et al., 2007), and *Griffithsia okiensis* (Lee et al., 2007).

These complete genome sequences and ESTs give perspectives on, respectively, what is possible from the full suite of genes in the organism, which can be recognized on the basis of known sequences, and what genes are being transcribed under the condition(s) to which the alga is exposed. Such studies are valuable in indicating the phylogeny of the organism and of component genes that could have arrived by endosymbiosis or horizontal gene transfer as well as by vertical transmission, although sequencing of many fewer genes can achieve robust phylogenies of the organism and of individual structural elements, of enzymes, transporters and light-harvesting and photochemical catalysts, and of metabolic pathways. Analysis of ESTs and, better, of transcripts using Q-RTPCR can give indications of the functioning of particular genes or pathways, although additional information from enzyme activity measurements and estimates of fluxes through the pathway may also be needed, especially when the gene product(s) could, or are known to, have functions in more than one metabolic sequence.

This chapter provides insights on how genomic studies have helped to determine what functional opportunities an organism has, and which of these possibilities are taken up under given environmental conditions, and where the relevant genes have come from. The chapter also points out where additional evidence is needed to determine which functions are occurring in the organism.

2. Fate of Genes from the Cyanobacterial Plastid Precursor in Red Algae

Since the discussion under this heading involves comparisons of the plastid endosymbiosis in red algae with the situation in other members of the Kingdom Plantae, it is necessary to consider the alternative possibilities for the endosymbiotic origin of plastids in the Plantae and, indeed, whether molecular genetics support this Kingdom using genes originating from the host, rather than cyanobiont plastid ancestor (Soon et al., 2008).

The most widely accepted view is that a single endosymbiotic event accounted for all of the "primary" plastids in the Plantae and, by secondary and tertiary endosymbiosis, for all plastids found in other eukaryotic clades (see discussion by Reyes-Prieto et al., 2007; Moore et al., 2008; Soon et al., 2008). A clear exception to this is the photosynthetic structure, derived from a very different cyanobacterium than the ancestor(s) of true plastids, in the euglyphid rhizarian *Paulinella* (Nowack et al., 2008). There is debate (Reyes-Prieto et al., 2007) as to whether this "cyanelle" is really a symbiont (Theissen and Martin, 2006) rather than an organelle (Bhattacharya and Archibald, 2006). Another exception is the intracellular, vertically transmitted cyanobacterium in the spheroidal body with the capacity to fix nitrogen in the diatom *Rhopalodia gibba* (Prechtl et al., 2004). This is clearly another independent origin of a cyanobacterial endosymbiont, and again it is not clear whether it fits the most rigid definition of a plastid- or mitochondria-like organelle (Prechtl et al., 2004; Larkum et al., 2007).

A second view is that there could be either two or three independent origins of "primary" plastids in accounting for the occurrence of such plastids in the Glaucocystophyta, Rhodophyta, and Chlorophyta plus Embryophyta (e.g., Stiller and Hall, 1997; Stiller et al., 2003; Stiller and Harrell, 2005; Larkum, 2007; Larkum et al., 2007; see also Soon et al., 2008).

A third possibility is a polysymbiosis of "shopping bag" model, involving genetic interchange at the origin of plastids (Larkum, 2007; Larkum et al., 2007).

While the majority are not always right, I shall assume that they are in this case for the rest of this chapter, and base the rest of the discussion on a single origin of "true" plastids.

Recent work analyzing 3,576 ESTs from the nuclear genome of the glaucocystophyte *Cyanophora paradoxa* revealed that 10.8% of the genes have come from the cyanobacterial plastid ancestor, and that only one-ninth of these code for nonplastid functions (Reyes-Prieto et al., 2006). Archibald (2006) found that the complete nuclear genome sequence of *Cyanidioschyzon merolae* (Matsuzaki et al., 2004) revealed a similar picture. This contrasts with flowering plants, where the corresponding fraction of the larger number of nuclear genes in *Arabidopsis thaliana* of cyanobacterial origin is 18%; this amounts to about 4,500 genes, of which about half have nonplastid functions (Reyes-Prieto et al., 2006). The most obvious explanation is that the plastid ancestor had at least 4,500 genes, and that 4,500 or more genes were present in the universal ancestor of plastid-containing eukaryotes with genetically integrated plastids to explain the number of cyanobacteria-derived genes found

in *Arabidopsis thaliana.* On this hypothesis, the early-branching algae lost many of the cyanobacterial genes involved in nonplastid functions present in the universal ancestor. Alternatively, subsequent horizontal gene transfer is required to account for the number of cyanobacterial genes in the flowering plants if an ancestor of these plants had as few cyanobacteria-derived genes as those found in the algae examined by Archibald (2006) and Reyes-Prieto et al. (2006). A further complication is evidence of a chlamydial symbiosis that facilitated the establishment of plastids from endosymbiotic cyanobacteria (Huang and Gogarten, 2007). There are also problems when secondary and tertiary endosymbioses are considered.

The red algae (like all eukaryotes) lack genetically integrated diazotrophy so that, if this was present in the cyanobacterial ancestor of the plastids, it has been lost. The same applies to gas vesicles. While the glaucocystophytes have retained the peptidoglycan wall of cyanobacteria around their plastids, and have what could be carboxysomes in the plastid stroma, although the molecular genetic evidence for this is by no means complete, these two structures are absent from red algal plastids. *Cyanidioschyzon merolae* lacks genes related to peptidoglycan synthesis, although some of these are found in flowering plants and an even greater number occur in the moss *Physcomitrella patens* where they are essential for chloroplast division (Machida et al., 2006). The genes involved in plastid (and mitochondrial) division in *Cyanidioschyzon merolae* are well characterized (Matsuzaki et al., 2004; Misumi et al., 2005).

The thylakoid reactions of *Cyanidioschyzon merolae* involve cyanobacterial genes, some of which are in the plastid genome and others have been transferred to the nucleus (Matsuzaki et al., 2004; Misumi et al., 2005). There is no gene coding for plastocyanin, so the only immediate electron to photosystem I is cytochrome c_6; also lacking are the genes of the NADH dehydrogenase complex (Misumi et al., 2005). As in cyanobacteria and most red algae, there are no genes related to xanthophyll cycles involved in nonphotochemical dissipation of excess excitation energy, neither is there the gene coding the protein that binds the energy dissipating form of the pigment (Misumi et al., 2005).

3. Plastid Functions in Red Algae Using Cyanobacterial and Noncyanobacterial Genes

Raven et al. (1990) review early evidence on carbon metabolism in red algal plastids, including the evidence that isolated plastids of red algae with very large cells are able to carry out all the reactions of photosynthesis, with the slight proviso that such plastids may encompass some cytoplasmic components and additional membranes when prepared from green algae.

Matsuzaki et al. (2004), Misumi et al. (2005), and Kitade et al. (2008) point out that the photosynthetic carbon reduction cycle enzymes of red algae, like those of other eukaryotes investigated, are a chimera of cyanobacterial and host eukaryote genes, some from the latter apparently of proteobacterial origin. Further complications

arise when enzymes catalyzing reactions of the photosynthetic carbon reduction enzymes in plastids have versions in the cytosol involved in glycolysis and gluconeogenesis are considered (Gross et al., 1999; Rogers and Keeling, 2004). The exception to vertical descent from the cyanobiont or the host among photosynthetic carbon reduction cycle is ribulose bisphosphate carboxylase-oxygenase (Rubisco). The cyanobacterial ancestor very probably had a form IB Rubisco, as do glaucocystophytes, green algae, and embryophytes, while red algae have a Form ID enzyme originating by horizontal gene transfer from a bacterium. The form ID Rubiscos typically have the highest reported selectivities for CO_2 over O_2, relatively high affinities for CO_2, low CO_2-saturated rates of carboxylation, and (probably) high selectivities for $^{12}CO_2$ over $^{13}CO_2$ (Tcherkez et al., 2006; McNevin et al., 2007). The extent of selectivity of carboxylases for ^{12}C over ^{13}C is probably an emergent trait (Raven, 1998a; cf. Shchepinov, 2007). Enzyme mechanistic determinants of the magnitudes of these four parameters are interdependent, such that it is not possible for a Rubisco to evolve, or be engineered, that would have a higher CO_2/O_2 selectivity combined with a higher CO_2-saturated rate of carboxylation (Tcherkez et al., 2006).

Despite these mechanistic constraints, modeling based on observed kinetic values suggests that substitution of the Form IB Rubisco in leaves of a C_3 land plant such as *Nicotiana tabacum* with equimolar quantities of the Form ID enzyme from the florideophyte red alga *Griffithsia monilis* would increase the rate of photosynthetic CO_2 assimilation at all of the chloroplast CO_2 concentrations modeled, from the CO_2 compensation concentration up to the concentration found with more than twice the present atmospheric CO_2 concentrations (Whitney et al., 2001). Such modeling exercises suggest that there is sufficient flexibility within the mechanistic kinetic constrains identified by Tcherkez et al. (2006) for there to be the potential for increased photosynthetic potential in organisms relying on diffusive entry of CO_2 in using Form ID and, especially, red algal Rubiscos (Whitney et al., 2001; Zhu et al., 2004; Galmes et al., 2005; Parry et al., 2007). There are, however, very significant technical difficulties with such replacement of a form IB with a red algal Form ID Rubisco (Whitney et al., 2001; Parry et al., 2007). Although practical outcomes of such enzyme replacements may be some way off, interest in this possibility has focused attention of the kinetic properties of red algal Rubiscos.

While the majority of the red algae that have been examined have inorganic carbon concentrating mechanisms (CCMs), a significant minority have diffusive CO_2 entry to Rubisco (Giordano et al., 2005; Raven et al., 2005). This is permitted by the high CO_2/O_2 selectivity and relatively high affinity for CO_2 of the Rubisco, coupled for some natural habitats with a low irradiance of photosynthetically active radiation, a rapid turbulent flow of water over benthic algae and/or a "subsidy" of CO_2 from catchment biogeochemistry (Raven et al., 2005). Diffusive CO_2 entry is more common in red algae, as a percentage of taxa tested for this trait, than in any algal clades other than the Chrysophyceae and Synurophyceae, which also have a Form ID Rubisco (Raven et al., 2005). In view of these differences in the availability of CO_2, especially taking into account the possibility that changes

in the inorganic carbon supply can be moderated in organisms with CCMs more readily than in those that cannot, it might be expected that there would be different selective forces acting on the Rubiscos of different red algae. Those with CCMs might be expected to emphasize the CO_2-saturated rate of carboxylation at the expense of the CO_2/O_2 selectivity or the CO_2 affinity, and vice versa for those lacking CCMs (Badger et al., 1998; Tcherkez et al., 2006). In view of this expectation, it is perhaps unexpected that Kapralov and Filatov (2007) found no detectable positive selection in the Rubiscos from the red algae that they examined, or indeed for any of the other algae or of secondarily aquatic monocotyledonous flowering plants, while a significant positive selection signal was found for flowering plants photosynthesizing with their shoots in the air. Kapralov and Filatov (2007) used nucleotide sequence analysis to determine whether positive selection had occurred; it would be useful to measure Rubisco kinetics for red algae lacking CCMs and those expressing CCMs. It is important to remember that the difference in activation energy for Rubiscos with the lowest and the highest known CO_2/O_2 selectivities is only about 6 kJ per mole, i.e., the energy of a single hydrogen bond (Spreitzer and Salvucci, 2002), so that there may not be the need for large changes in nucleotide sequence to account for ecologically very significant changes in enzyme kinetics.

There is no gene coding for Rubisco activase in *Cyanidioschyzon merolae* or in any organism outside the Chlorophyta (and plastids derived green algae by secondary endosymbiosis: Durnford and Gray, 2006) and Embryophyta, with the probable exception of some multicellular (Kaiser, 2001) cyanobacteria (Li et al., 1993; Pearce, 2006). The role of the ATP-consuming activase is to remove tightly bound inhibitors that are produced directly from intermediates of the catalysis, or are produced by other reactions from these initial products. It seems that the Form II Rubisco of the proteobacterium *Rhodospirillum rubrum*, the Form IB Rubisco of the cyanobacterium *Synechococcus* PCC6301, and the Form ID Rubisco of *Galdieria sulphuraria*, while forming by-products, no inhibition was found at substrate saturation (Pearce, 2006). The three Rubiscos have different mechanisms, which restrict inhibition by these by-products, and which can explain why Rubisco activase is not required, whereas activase is required by the high plant (and green algal, including algae that obtained chloroplasts from green algae in secondary endosymbiosis) Form IB Rubiscos (Pearce, 2006).

The regulation of the photosynthetic carbon reduction cycle enzymes in the red alga *Galdieria sulphuraria* has been shown to differ from that in the green alga – embryophyte clade, with attempts to relate the differences to changed nucleotide sequences in the genes (Reichert et al., 2003; Oesterhelt et al., 2007). This work shows that there is less emphasis on regulation by redox state in the cases of phosphoribulokinase (PRK) and of fructose-1,6-bisphosphate-6-phosphatase, and an important role for the association of PRK and of NADP-glyceraldehyde-3-phosphate dehydrogenase with the protein CP12 for inactivation in the dark.

Turning now to the fate of the photosynthate, the plastid of *Galdieria* contains one of the two isoforms of glucose-6-phosphate dehydrogenase, the first enzyme of the oxidative pentose phosphate pathway (Oesterhelt et al., 2007;

Tischendorf et al., 2007). This enzyme is inactivated by light by reduction and is reactivated by oxidation at the beginning of darkness (Oesterhelt et al., 2007).

Export of photosynthate from plastids in the cyanidiophyceans involves the triose phosphate (or phosphoglycerate) – phosphate antiporter in the inner plastid envelope membrane (Barbier et al., 2005) found in chloroplasts from other organisms. These organisms lack the dicarboxylate (oxaloacetate – malate) antiporter in the inner envelope membrane, and also lack the plastid-located NADP-dependent malate dehydrogenase, which is the other component of the "malate valve" (Barbier et al., 2005; Oesterhelt et al., 2007). It is not clear what the implications of this are for the redox balance of the chloroplasts and cytosol. The malate valve is essential for photorespiratory metabolism in C_3 flowering plants; the implications of this for cyanidiophytes (and other red algae) is not clear in view of uncertainties as to the pathway(s) involved and the location of the individual reactions, as well as its quantitative significance in view of the kinetic properties of Form 1D Rubiscos and the frequent occurrence of CCMs. This is considered under "Mitochondria" and "Peroxisomes."

Starch in green algae and embryophytes occurs in plastids, whereas in all other starch-storing algae (glaucocystophytes, rhodophytes, cryptophytes, and dinophytes), the starch is in the cytosol (Raven, 2005). Patron and Keeling (2005) showed that despite the different intracellular compartmentation of the starch biosynthetic enzymes in green and in red algae, all but one of the enzymes shares a common origin in the two groups. The starch synthetic enzymes have mixed host and cyanobacterial origins, so there are host-derived genes functional in the plastids of red algae and cyanobacterium-derived genes in the red algal cytosol. Further investigation is needed for the ancestry of the starch-metabolizing enzymes in other clades, as well as the molecular basis of starch metabolism in plastids and in the cytosol (Raven, 2005).

Low molecular mass compatible solutes and/or organic carbon storage compounds in the red algae show great diversity, with floridoside and isofloridoside occurring very widely, with digeneaside and a number of sugar alcohols in some clades. The metabolism of sugars and related compounds in cyanidiophytes is considered from a genomic perspective by Barbier et al. (2005) for the facultatively chemo-organotrophic *Galdieria sulphuraria* and the obligately photolithotrophic *Cyanidioschyzon merolae*. It is still not clear how osmotic and volume regulation is achieved among the various intracellular compartments, including the chloroplasts, of red algae.

Sato and Moriyama (2007) have examined the fatty acid composition, and the genes involved in the synthesis of fatty acids, in *Cyanidioschyzon merolae*. This thermoacidophilic alga lacks fatty acids with three or more double bonds; while this could be related to the high temperature of this alga's habitat, other red algae also have a limited quantity of polyunsaturated fatty acids. The *Cyanidioschyzon merolae* genome lacks the acyl lipid desaturases derived from the plastid ancestor, as well as the stearoyl acyl carrier protein desaturase; these are major desaturases in green algae and plants. Labeling shows that desaturation occurs outside the plastid, so that the production of polyunsaturated fatty acids involves desaturation on the endoplasmic reticulum of palmitic acid synthesized in the plastids.

Carotenoid biosynthesis in *Cyanidioschyzon merolae* has been examined by Cunningham et al. (2007). This alga has a very restricted range of carotenoids relative to any other red algae, i.e., β-carotene and zeaxanthin. While the genes for the pathway from isopentyl diphosphate and dimethylallyl diphosphate to β-carotene were identified, there is no indication of how β-carotene is hydroxylated.

Much of the tetrapyrol synthesis pathway in algae and plants occur in plastids; the enzymes have a mixed origin; not all of them come from the cyanobacterial plastid ancestor (Oborník and Green, 2005). Red algae are distinctive in having a ferrochelatase gene different from that of cyanobacteria and other plastids (including those derived by secondary endosymbiosis from a red alga), apart from those (derived from a red alga) of apicomplexans. The red algal ferrochelatase gene is of proteobacterial origin.

4. Mitochondria Functions

Genome sequences of *Cyanidioschyzon merolae* and *Galdieria sulphuraria* show the presence of the expected ATP–ADP and 2-oxoglutarate-malate antiporters, and a range of protein import-related components, of the mitochondrial membranes, as well as a trio of mitochondrial division mechanisms (Matsuzaki et al., 2004; Barbier et al., 2005; Misumi et al., 2005). EST analysis of *Porphyra haitanensis* showed the presence of a mitochondrial alternative oxidase (Xiaolei et al., 2007). The occurrence of an alternative oxidase in *Chondrus crispus* was suggested by the *in vivo* inhibitor experiments of Furbank and Rebeille (1986), although alternative oxidase inhibitors also inhibit lipoxygenases (Bourareb et al., 2004). Furbank and Rebeille (1986) found a residual respiration with a low affinity for O_2 after cytochrome oxidase had been inhibited by KCN and the alternative oxidase (and lipoxygenase) had been inhibited by salicylhydroxamic acid (see Raven et al., 1990).

Enzymes of the photorespiratory carbon oxidation cycle are best characterized from vascular plants; here, some enzymes occur in the plastids, others in peroxisomes, and yet others in mitochondria (Igamberdiev and Lea, 2002). The situation in most algae, including red algae, is much less clear. Stabenau and Säftel (1992) isolated mitochondria and peroxisomes from *Erythrotrichia carnea*: the mitochondria have a glycolate dehydrogenase, which also oxidizes D-lactate, in contrast with the L-lactate-oxidizing glycolate oxidase in the peroxisomes. No serine hydroxymethyltransferase activity was detected in the mitochondrial fraction, although that is where it occurs in vascular plants. The gene for the p-subunit of glycine decarboxylase has been found as an EST in *Griffithsia okiensis* (Lee et al., 2007). The ammonia released by mitochondrial glycine deacroxylase in land plants is recycled into glutamine by glutamine synthetase in the chloroplasts or, as has recently been found, the mitochondria (Link and Weber, 2005); the cytosolic form of the enzyme is involved in other ammonia assimilation reactions. In *Galdieria sulphuraria*, there is only a cytosolic form, which must be involved in any ammonia reassimilation that is needed in photorespiration (Terashima et al., 2006).

5. Peroxisome Junctions

Peroxisomes differ from plastids and mitochondria in lacking a genome and not originating by endosymbiosis. *Cyanidioschyzon merolae* has only one peroxisome per cell, and these undergo binary fission at cell division, although a key gene involved in microbody division is not present in the genome (Misumi et al., 2005). The *Cyanidioschyzon merolae* genome has one of the peroxisomal protein-import mechanisms but lacks the other (Misumi et al., 2005). There is also a gene for a peroxisomal catalase.

Stabenau and Säftel (1992) isolated peroxisomes of the red alga *Erythrotrichia carnea* and showed that they contained glycolate oxidase and hydroxypyruvate reductase as well as catalase, which breaks down H_2O_2 produced by (for example) glycolate oxidase. Another enzyme that oxidizes glycolate is found in mitochondria; this enzyme is a dehydrogenase that also oxidizes D-lactate, whereas the peroxisomal glycolate oxidase also oxidizes L-lactate. Seckbach et al. (1992) found glycolate oxidase in peroxisomes of cyanidiophyceans, but serine-glyoxylate aminotransferase and hydroxypyruvate reductase were in the cytosol. The pathway(s) of metabolism of glycolate produced by Rubisco oxygenase in red algae awaits resolution (see Eisenhut et al., 2006 for work on cyanobacteria). Although the Form ID Rubisco of red algae produces less phosphoglycolate under a given set of conditions than almost any other Rubisco, there is still a need for metabolism and/or excretion of glycolate even when there is a CCM (Giordano et al., 2005).

6. Absence of Flagella

Production, maintenance, and operation of flagella, another structure that is not generally considered to be derived from symbiosis, can account for more than 1% of the resource costs of cells (Richardson and Raven, 1984). Red algae have no flagella, and so avoid these costs. Despite the absence of flagella on their spores or male gametes, red algae achieve dispersal and fertilization to extents comparable with other macroalgae (Hawkes, 1990; cf. Raven, 1998b; Engel et al., 1999; Fierst et al., 2005). Wickstead and Gull (2007) present a comparative genomic analysis of the distribution of dyneins, the mechanochemical transducers of flagella movement and also functional in intracellular transport in many eukaryotes. They point out that *Cyanidioschyzon merolae* lacks axonemal dyneins and, indeed, all but one of the cell body dyneins, so (this) red alga(e) lack(s) the cost of maintaining at least the dynein components of the flagella as well as the costs of expressing the genes.

7. C_4 Photosynthesis in Red Algae?

Xiaolei et al. (2007) suggested that the bangiophyte *Porphyra haitanensis*, which had already been shown to have a CCM (Zou and Gao, 2002a, 2002b, 2004), has C_4 pathway are transcribed in *Porphyra haitanensis*, and Derelle et al. (2006) suggested that the green (prasinophyte) alga *Ostreococcus tauri* could have C_4

photosynthetic metabolism, based on the complete genome sequence. However, all of the enzymes needed for the PEPCK-based C_4 pathway, as well as those used in the NAD-malic enzyme and NADP-malic enzyme C_4 variants, are found in flowering plants, which are well established to have C_3 photosynthetic biochemistry and physiology (Ku et al., 1996; Schultz et al., 1998; Wheeler et al., 2001a, b; Jenner et al., 2005; Malone et al., 2007). Furthermore, Ku et al. (1996) comment that C_4 flowering plants have specific C_4 isoforms of the C_4 enzymes in addition to the isoforms used in metabolism other than C_4 photosynthesis, although it may be possible for effective C_4 photosynthesis to occur using the same enzyme isoforms as those used for other aspects of metabolism.

Other relevant data come from short-term inorganic ^{14}C labeling experiments, from which the conclusion for seaweeds (brown, green, and red) other than the ulvophycean green alga *Udotea flabellum* is that photosynthesis involves C_3 biochemistry, with some hints of C_4 physiology in some organisms under some circumstances (Kremer and Küppers, 1977; Johnston, 1991; Raven, 1997). Such labeling data, preferably with the shortest labeling time of 1–2 s, are needed for *Porphyra haitanesis* as well as for other red algae. In this context, work of marine diatoms is relevant. While the complete genome sequence of *Thalassiosira pseudonana* would permit the PEPCK, or NADP-malic enzyme, variants of C_4 photosynthesis to occur, the inorganic ^{14}C labeling and transcriptomic data suggest C_3 biochemistry (Roberts et al., 2007), though some inhibitor data (McGinn and Morel, 2008) are difficult to reconcile with C_3 photosynthetic biochemistry. Genomic data for the diatom *Phaeodactylum tricornutum* is also not in good agreement with C_4 photosynthesis in this alga either (Kroth et al., 2008; cf. McGinn and Morel, 2008).

We must not underestimate the significance of EST (and, more generally) genomic studies; however, the example that has just been discussed shows that alternative explanations, and the need for additional work using other techniques, must be borne in mind. Finally, it must be admitted that this analysis is asymmetric. This is because the alternative, or adjunct, to C_4 metabolism in constituting a CCM is active transport across one or more membranes of one or more of bicarbonate, carbon dioxide, and protons, and the components of such mechanisms are less well known in molecular genetic terms than the components of the C_4 pathway (Giordano et al., 2005).

8. Conclusions

Genomics have been very influential in telling us about the evolutionary history of genes in the red algae, and has indicated the chimeric nature of many metabolic pathways. As with other organisms, genomic studies on red algae show what functions are possible in that organism. However, genomic studies do not necessarily reveal which pathways are actually used by the organism, even when the genes are known to be transcribed and translated: this is especially the case if the proteins can function in more than one process.

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10. Note Added in Proof

Since the manuscript was accepted in 2008 there have been many advances in the fields that it covers. Some of these are covered by Raven et al. (2009) and Raven (2010).

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COMPUTATIONAL GENE PREDICTION IN EUKARYOTIC GENOMES

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1. Introduction

The number of sequenced eukaryotic genomes is increasing faster owing to rapid advances in DNA sequencing technology. After a new genome has been assembled into contigs or chromosomes, a first crucial task is to identify the location and structure of its protein-coding genes. This task requires one to find the precise splicing patterns of genes including all introns and exons (cf. Fig. 3) and therefore entails in particular finding the amino acid sequences of all proteins encoded in the genome. Because of the large amount of genomic data, in silico methods are needed for this genome annotation task. Consequently, genome sequences are annotated using mostly computational gene prediction programs.

Gene prediction approaches can be divided into two major categories, ab initio approaches and *extrinsic* approaches. Ab initio gene prediction programs try to find the genes solely based on the target DNA sequence, i.e., the DNA sequence in which the genes need to be identified. The parameters of the programs may be determined using known genes from the target genome. For the prediction, however, ab initio programs use only the target DNA sequence. Extrinsic approaches use additional (extrinsic) data as evidence when predicting the genes. These data are most commonly cDNA sequence data, the protein sequences of known genes in related species, or the genome sequences of species closely related to the target species. Approaches using only the later evidence, i.e., the unannotated genomes of closely related species, are called de novo or comparative approaches. Depending on the available extrinsic data, extrinsic approaches can be significantly more accurate on average than ab initio approaches. However, in most genome projects, at least a part of the gene structures are partially unsupported by extrinsic data, so that ab initio methods are still required.

Unfortunately, for most eukaryotic genomes the gene finding task turns out to be very hard and the currently best available programs still predict many gene structures incorrectly. The recent independent assessment EGASP of state-of-the-art gene finders in human had the result that in the complex human genome, only 40–50% of transcripts (including alternative splice variants) are predicted correctly by the best programs using all available information (Guigó et al., 2006). Another recent independent assessment, nGASP, of gene finders on the less complex genome of *Caenorhabditis elegans* had the preliminary results that each of the best gene finders predicts only 86% of the exons correctly (Coghlan et al., 2008).

Given the imperfection of current gene predictors, improvements in general applicable gene finding methodology are an essential and direct advantage for hundreds of eukaryotic genome projects with diverse applications in biology, medicine, biotechnology, and agriculture. Thus, the discipline of computational gene finding remains a highly competitive field in Bioinformatics. In this chapter, we give an overview of the current main approaches in this field with particular reference to red algae. For recent reviews on gene finding focusing on Eumetazoa see Brent (2008), Guigó et al. (2006), or Brent and Guigó (2004).

2. The Significance of Intron Distributions for Gene Prediction

In contrast to prokaryotes, in which each gene is encoded by a contiguous segment of DNA, most eukaryotic genomes contain introns, which interrupt the genes. However, there also exist a few eukaryotes in which most genes are intronless such as the red alga *Cyanidioschyzon merolae* (Nozaki et al., 2007) and the yeast *Saccharomyces cerevisiae*.

In prokaryotes and in eukaryotes with few introns, the genes can be identified with fairly high accuracy. The locations of genes can be derived with relatively high confidence by searching for long open reading frames (ORFs) possibly also supported by a database similarity search. The longest ORF in a given region is very likely to be protein coding as it would otherwise have acquired mutations that introduce an in-frame stop codon somewhere. The research in prokaryotic gene finding therefore seeks to improve methods for identifying the precise translation start sites given the gene (Tech and Meinicke, 2006).

Gene prediction in eukaryotic genomes, which contain a substantial amount of introns, requires generally different approaches. The necessity to detect such splicing is the main reason why gene prediction is generally much more difficult in eukaryotes. The rest of the chapter will describe the issues for such genomes. In eukaryotic genomes, the frequency of multi-exon genes and the length distribution of introns determine to a large extent how difficult it is to identify the genes of that species and therefore how accurate current gene finding methods are.

The intron length distributions vary strongly from species to species. Vertebrates tend to have the longest introns. For example, the average human intron is about 5,400 base pairs (bp), about 10% of introns in the human genome are longer than 11 kilo base pairs (kb), and a few introns are even close to 500 kb long (Sakharkar et al., 2004). In *Arabidopsis* and rice, the average intron lengths are 173 and 433, respectively, and the percentage of introns longer than 1 kb are 0.8% and 10.4%, respectively (Wang and Brendel, 2006). In the red alga *G. sulphuraria*, almost all introns have a length very close to the average length of 53 bp and only very few introns are longer than 100 bp (see Fig. 1).

Bernal et al., examined how accurate a group of successful ab initio gene finders is in identifying human introns (Bernal et al., 2007). They report that the probability, that an intron is predicted correctly, decreases significantly with

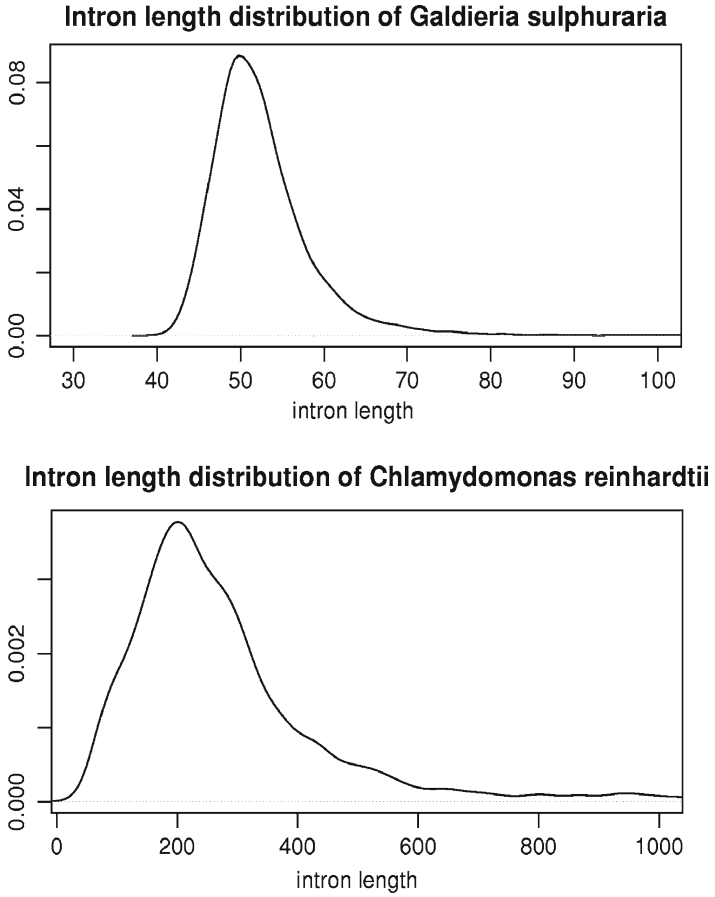

Figure 1. Intron length distributions of two algae. The intron lengths of *G. sulphuraria* have an exceptionally low variation as most of them are short. 85% of introns have a length in the narrow range between 45 and 58 bp. Only 0.5% of introns are longer than 100 bp. For comparison, we show the intron length distribution of the green alga *C. reinhardtii*, which shows higher variation and is more typical of other eukaryotes.

the length of the intron. This effect can be explained as follows. All ab initio methods sometimes predict false-positive exons, i.e., they erroneously label a sequence interval as an exon, that is not an exon. A particularly long intron is therefore hard to predict correctly as there is a relatively high chance that some subinterval of that intron is mistaken as an exon.

Shorter introns also limit the complexity of the gene structures and the types of alternative splicing that can be observed in a genome. In the case of *G. sulphuraria*, where almost no introns are longer than 100 bp, nested genes are impossible given this constraint. However, nested genes do occur in more complex genomes such

as *C. elegans* and human. A common form of alternative splicing are so-called "cascade exons." These are exons that are completely skipped in an alternative splicing pattern. Since in the transcript variant where the exon is skipped, one intron has a length that equals the length of two introns and the cascade exon together, this is also virtually impossible in a genome such as *Galdieria* where almost all introns have approximately the same length.

3. Ab Initio Gene Finding

3.1. MODELS AND PROGRAMS

The development of ab initio models for gene finding is important mainly for two reasons. First, they are needed to localize the genes for which not sufficient extrinsic information is available. This is the case for genomes, for which no closely related genome has been sequenced yet and for genes in that genome which do not show sufficient similarity to known proteins in the databases and for which no cDNA data are available. The second motivation for ab initio gene modeling is that such models, or parts of the models, can also serve as base for extrinsic gene finding methods. In fact, this is frequently the case for de novo methods, which exploit the conservation information between several unannotated genomes (Stanke et al., 2006).

In the models of gene finders, a *gene structure* of a DNA input sequence is usually interpreted as a labeled segmentation $g = (g_1, g_2,..., g_n)$ of the input sequence s. Let $g_i = (a_i, b_i, \lambda_i)$. Each segment from sequence position a_i to b_i is assigned one biological label λ_i. Exactly which labels are chosen and the extent and interpretation of the segments depends on the particular program. However, ab initio gene finding methods for eukaryotes have in common that they employ machine learning methods to score or recognize (1) biological signals such as the translation start site, the donor (5′) splice site or the acceptor (3′) splice site and (2) labeled regions such as coding exons, introns, and intergenic regions (Table 1).

Table 1. Example of a gene structure g.

	Position		**Biological label**
	From	**To**	
I	a_i	b_i	λ_i
1	1	2,765	Intergenic region
2	2,766	2,787	Translation start site signal
3	2,788	2,913	Initial coding exon
4	2,914	2,922	Donor splice site signal
5	2,923	3,461	Intron
6	3,462	3,468	Acceptor splice site signal
7	3,469	3,577	Internal coding exon

As no known machine learning methods are currently known to detect such biological signals or regions isolatedly and with confidence, ab initio gene prediction programs use the fact that the overall confidence can be increased when the combination of biological signals, exon and intron scores, and other features of a gene structure are considered simultaneously. The models define a score or probability of a gene structure as a function of the scores or probabilities of the individual segments in the candidate gene structures.

For example, if a so-called hidden Markov model (HMM) is chosen as a modeling class, the probability of a gene structure g given the input DNA sequence s is typically of the form

$$P(g \mid s) \propto \prod_{i=1}^{n} P(\lambda_i \mid \lambda_{i-1}).\, P(s[a_i, b_i] \mid \lambda_i) \tag{1}$$

Here, the factors $P(\lambda_i \mid \lambda_{i-1})$ are the probabilities for a transition from one biological label λ_{i-1} to the next biological label λ_i (To simplify notation, let λ_0 be a special label "start of sequence."). In particular, these transition probabilities determine which biological labels may follow which in order for g to be a valid gene structure. The model is termed *HMM* because the sequence λ_1, λ_2, ... forms a so-called Markov Chain and because the λ_i's are "hidden" from observation. The factors $P(s[a_i,b_i] \mid \lambda_i)$ are the probabilities of the ith DNA segment given that it is labeled λ_i. Modeling the details of these probabilities allows one to specify, e.g., what a typical exon sequence looks like; that there must be no in-frame stop codon in an exon; that the coding sequence should start with ATG; or that an acceptor splice site should have the dinucleotide consensus AG.

Usually, the programs then use a dynamic programming algorithm to find the best gene structure g for the input sequence under this model. In the case of HMM-based gene finders, usually the gene structure g that maximizes $P(g \mid s)$ is computed using a variant of the so-called Viterbi algorithm.

While early efforts in computational gene finding date back to 1982 (Staden and McLachlan, 1982), complete gene finders in the above sense were introduced in the 1990s. The program GENMARK (Borodovsky and McIninch, 1993) was the first gene finder that uses a Markov model for the coding regions. This is still the most prevalent model class in current gene finders. GENIE (Kulp et al., 1996) and GENSCAN (Burge and Karlin, 1996) were among the first gene finders for eukaryotes based on an HMM. For more than 10 years, and only until recently, the best-performing ab initio gene finders were based on an HMM. Important HMM-based gene finders are SNAP (Korf, 2004) and the ab initio versions of TWINSCAN (Korf et al., 2001) and AUGUSTUS (Stanke and Waack, 2003). GENSCAN had been the most accurate ab initio gene-finding program for human for many years after 1996 and was later outperformed by AUGUSTUS and other programs (Guigó et al., 2006). Recently, the more general model class of conditional random fields (CRFs) has been introduced. CRFs are discriminative rather than generative models and the CRF-based

program CRAIG has currently the best published accuracy results for human (Bernal et al., 2007).

Ab initio programs are widely applicable as they do not require further data. However, they suffer from a number of limitations. With few exceptions, ab initio programs predict only the coding part of a gene. The untranslated regions (UTRs) are usually ignored and considered to be "intergenic region." However, there exist many programs that try to identify promoters (Bajic et al., 2006). AUGUSTUS predicts 5′-UTR and 3′-UTR including introns only for a subset of the species it is trained for.

Another limitation is that almost all ab initio programs can currently only predict one transcript per gene. Exceptions are HMMgene (unpublished) and AUGUSTUS (Stanke et al., 2006a, b). The targeted prediction of alternative splicing with ab initio methods is currently rather unreliable or limited to certain types of alternative splicing (Allen and Salzberg, 2006).

3.2. TRAINING OF PARAMETERS

Fortunately, the gene organizations of different eukaryotes are so similar that a sufficiently general gene finding approach can in principle be applied to almost any eukaryotic genome. However, for optimal performance, gene finders need to be *trained* for a given target species or clade of species. "Training" here means that the numerous parameters of the model are estimated or adjusted for the gene prediction to perform well on the target species. Such a training is usually not necessary for purely expression-based methods that predict transcripts only in regions where they are supported by alignments to known proteins and/or cDNA (Slater et al., 2005). However, in particular ab initio and de novo models can perform poorly if they are applied to a species for which they were not properly trained for (Korf, 2004).

A survey of currently available gene finders and foreign-trained parameter sets yields that they do not perform well on *G. sulphuraria.* For example, the program GeneID (Parra et al., 2000) has been trained for the four protists *Dictyostelium discoideum*, *Plasmodium falciparum*, *Perkinsus marinus*, and *Trypanosoma brucei*. When these four versions of GeneID are used to predict the genes in *G. sulphuraria*, the fractions of exons that are predicted correctly are 4%, 0.7%, 8%, and 0.4%, respectively. This performance is very low (when compared with Fig. 3), although GeneID belongs to the programs that perform among the best on species it is trained for, e.g., in humans (Guigó et al., 2006). Similarly, the best performing foreign parameter set of AUGUSTUS is that of the ciliate *Brugia malayi.* However, only 21% of exons in *G. sulphuraria* are predicted correctly using this parameter set. These results suggest that in the case of *G. sulphuraria*, cross-species gene prediction is very likely to be useless, possibly because the features in the foreign genomes that are used for gene finding are too different from the ones in this red alga.

The reason for a bad cross-species gene prediction performance is that the statistical patterns at biological signals, the base composition of the genome, the

Figure 2. Acceptor splice site profiles of *G. sulphuraria* (*top*) and *C. reinhardtii* (*bottom*). The figure shows the frequencies of the nucleotides in the window [−17, 3] relative to the 3′ splice site of the red alga *G. sulphuraria* and the green alga *C. reinhardtii.* The two algae show distinct patterns. For example, cytosine is the most frequent base in *C. reinhardtii* at most of the 3′-end intron positions before the AG dinucleotide consensus. However, at the same time, it is the least frequent base in many of the corresponding positions in *G. sulphuraria.*

codon frequencies, the intron length distributions, and other typical parameters of the models vary substantially from species to species. Figure 2 exemplifies this by comparing the frequencies of bases around the exon/intron boundaries of the red alga *G. sulphuraria* and of the green alga *C. reinhardtii*.

Many gene prediction programs were initially trained by the authors for only some species and cannot be retrained easily by users for new species. Gene finders that include programs for automatic retraining on a new genome are AUGUSTUS, SNAP, and GeneMark.HMM (Lomsadze et al., 2005), which are also examples for the following three different approaches to training.

3.2.1. Parameter Estimation on a Native Training Set

The traditional method for retraining a gene finder on a new genome is to estimate its parameters on a relatively small set of bona fide genes from the target species. Such training sets have been constructed using different methods. If ESTs are available for the target genome, they can be clustered and aligned to the genome assembly to yield a subset of gene structures of highly expressed genes. Another approach is to use protein-based gene prediction or alignment methods to identify the gene structures of genes that have high similarity to known proteins (Parra et al., 2007). Further, training sets can be prepared by manual inspection of cDNA and protein alignments. Besides the value in training gene finders, a further advantage of having a set of bona fide gene structures for the target genome is that this gene set can also be used to estimate the accuracy of gene finders and to compare their relative performance.

3.2.2. Cross-Species Training

In light of the fact that for many newly sequenced genomes, neither a training gene set nor a well-performing foreign-trained gene finder exists, Korf suggested

and analyzed a cross-species training procedure (Korf, 2004). The underlying idea is that a foreign-trained gene finder may perform well enough for its predictions in the target genome to serve as a training set. Even though this predicted training set may contain a significant amount of errors, it can still be sufficiently accurate so that the subsequent training on this set captures the species-specific features. The predictions of the gene finder that is trained on the predicted genes may then be more accurate than these predicted genes it was trained on. It should be noted that – somewhat counterintuitively – not always the parameters of the closest related species yield the best accuracy results in cross-species prediction.

With such an indirect parameter estimation approach, Korf reports good accuracy results in *Arabidopsis thaliana* for example when using *C. elegans* parameters to train his gene finder SNAP for *A. thaliana.* In the particular case of *G. sulphuraria* however, this bootstrap training approach would very likely fail because of the above-mentioned poor initial performance of foreign gene finders.

3.2.3. Iterative Self-Training

Recently, an idea for an unsupervised training procedure has been proposed that does not require a training set and can estimate the statistical models directly from unannotated genomic DNA. Lomsadze et al., take the following iterative self-training approach implemented in the program GeneMark.hmm (Lomsadze et al., 2005). The algorithms starts off with a "weak" initial model for genes, that is simple and depends only on the G+C content of the genome. At each step, the model with the current parameters is used to predict the genes on the input genome. This segmentation of the genome into coding and noncoding regions is then used to reestimate the parameters of the model. This iterative process of prediction and training is repeated until the gene predictions "converge" and do not change much from one step to another. Using this unsupervised training procedure, Lomsadze et al., report accuracy results for several model organisms that are approximately in the range of supervised training procedures, which use a training set. Sometimes, the results using the unsupervised training are even more accurate than those of the supervised training. This iterative self-training algorithm is a promising approach for training ab initio gene finders, in particular in "naked" genomes without extrinsic evidence. However, Lomsadze et al., anticipate some difficulties for some genomes of both low and high eukaryotes.

4. Extrinsic Approaches to Gene Finding

Many approaches have been developed to predict genes based on extrinsic evidence and to improve the accuracy over ab initio methods. The most commonly used evidence sources are cDNA sequences of the target species and protein sequences of related species. Such transcript-based approaches are described below in 4.1 and 4.2. Another source of extrinsic evidence that is incorporated by a large number of recent programs are genomes that are closely related (but not too closely) to the target

genome. These de novo methods either simultaneously predict the genes and align two syntenic genome sequences or take a given (multi)genome alignment and exploit the conservation information to predict the genes in the target genome. Recently, the de novo gene finder CONTRAST (Gross et al., 2007) has been introduced that has a significantly higher accuracy than previous de novo methods. This approach promises to be an important methodology for groups of relatively closely related genomes, e.g., mammals or flies, for which already numerous sequenced genomes exist. Unfortunately, de novo gene finding is likely not (yet) relevant for algae, as the genomes must be closely related enough to allow genomic alignments. Another source of extrinsic information that has recently been used in gene finding is peptide mass spectrometry (Tanner et al., 2007), which validates gene prediction at the level of translation. Further, in many recent genome projects, so-called combiner programs are used to call the final gene models. These programs combine gene predictions of several other programs with extrinsic evidence from cDNA and proteins. The most successful combiner-type program is JIGSAW (Allen and Salzberg, 2005).

4.1. USING CDNA ALIGNMENTS

Among the most reliable sources of experimental evidence for a gene structure are full-length mRNAs of the studied organism itself that can be aligned to the genome. However, much more abundant in the public databases are expressed sequence tags (ESTs), which constitute a random single-read fragment of cDNA. ESTs that have been sequenced using the traditional Sanger sequencing method are up to 700 bp long, the average being roughly 500 bp. Newer, high throughput pyrosequencing methods such as the technology of 454 life sciences generate shorter reads but at dramatically lower costs per sequenced base pair (Margulies et al., 2005). Therefore, this data source for genome annotation can be expected to gain importance in the future.

The appropriate method for aligning these cDNA sequences to a genome is a so-called *spliced alignment* program. The term "spliced alignment" refers to the fact that long insertions in a cDNA-to-genome alignment usually correspond to introns. Established spliced alignment programs for aligning cDNA to a genome are for example BLAT (Kent, 2002) and sim4 (Florea et al., 1998). The program PASA (Haas et al., 2003) assembles spliced alignments into clusters that constitute longer transcript fragments. The well-known BLAST suite (Altschul et al., 1990), however, is not well suited for aligning cDNA in the presence of introns because BLAST does not explicitly take introns into account and the high scoring sequence pairs correspond therefore only roughly to exons.

EST sequences are of relatively low quality, short and each sequence usually covers only a small part of the transcript. Some ESTs sequences still contain the intronic sequence. Despite these deficiencies, EST data helps significantly improving the accuracy of computational genome annotation. Purely expression-based methods that use cDNA alignments are for example the UCSC Known Genes

pipeline for human, mouse and rat (Hsu et al., 2006), and AceView (Thierry-Mieg and Thierry-Mieg, 2006).

Widely applicable gene prediction programs that can integrate evidence from cDNA alignments but do not require a full coverage of the gene with cDNA are AUGUSTUS (Stanke et al., 2008), N-SCAN_EST (Wei and Brent, 2006) and the commercial program Fgenesh (Solovyev et al., 2006).

Figure 3 shows an example of how EST data help in correcting gene models. The genome browser screenshot shows EST alignments and the ab initio and EST-supported predictions of AUGUSTUS in a region of the *G. sulphuraria* genome. The ab initio prediction is likely to be incorrect as the evidence from the ESTs suggests that one of the predicted introns is not present. The predicted coding region therefore changes frame, skips a stop codon, and two genes are joined and predicted as one gene. Using the ESTs as extrinsic evidence corrects this error and therefore two genes are predicted. Note that even although the coverage of the genes with ESTs is exceptionally high in *G. sulphuraria*, EST alignments by themselves are not sufficient to uncover the complete gene structure of the genes in Fig. 3. Ab initio methods are at least required to predict the gene structures in the areas lacking extrinsic evidence.

Figure 4 shows how much EST data (from 454 pyrosequencing) helps in increasing the accuracy of gene structure annotation in *G. sulphuraria*. As can be expected, the relative gain in accuracy, when sequencing and incorporating in the predictions increasing amounts of cDNA data, is initially higher and then levels

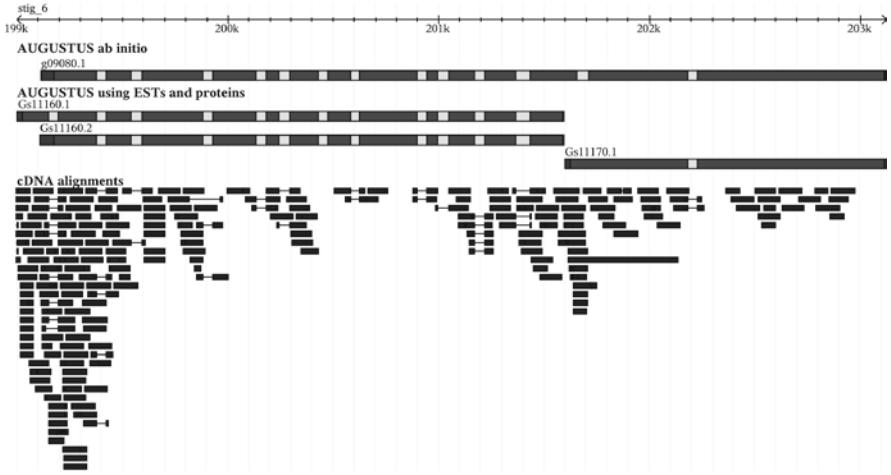

Figure 3. Example of a gene prediction in the red alga *Galdieria sulphuraria.* The ab initio gene track on the top predicts one gene on the reverse strand. The predictions on the second track are mainly based on the cDNA alignments and encompass two genes, one of which has two alternative transcripts. Introns are depicted in light gray, exons in darker gray. The bottom track shows alignments of cDNA sequences against the genome. Thin horizontal lines in the alignments correspond to gaps and suggest introns.

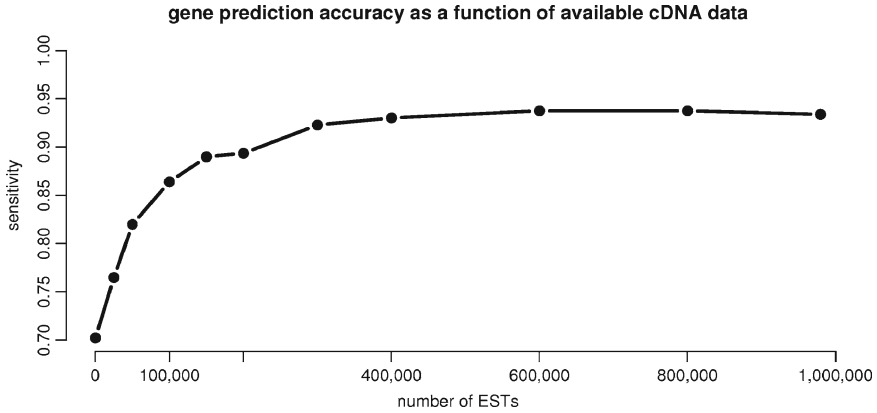

Figure 4. Accuracy in *G. sulphuraria* versus the number of used cDNA sequences. The graph shows the accuracy of the gene predictions of AUGUSTUS when different amounts of *G. sulphuraria* cDNA data are used as extrinsic evidence. The vertical axis shows the fraction of internal and terminal coding exons that were correctly predicted at both ends. Methods: The total number of available cDNA sequences for the 13.7 Mb genome was 979646, the average length being 99 bp. The subsets of the different sizes were chosen randomly under the uniform distribution to simulate smaller amounts of available cDNA data. For the empty subset of cDNA sequences at position 0, the ab initio model was used.

off due to the saturation effect of resampling previously sequenced cDNA. At about 300,000 ESTs, the additional gain in accuracy becomes marginal. This point corresponds to a ~2.8-fold coverage of the transcriptome with cDNA.

4.2. USING PROTEIN HOMOLOGY

Whereas cDNA data are informative only when it stems from the target organism itself or from a close relative, the incorporation of protein homology data is beneficial also for more distantly related species. This is due to the fact that conservation on the protein level can easily be detected than the genome-level conservation of the same gene.

One of the most established gene prediction programs that use protein homology information is GeneWise (Birney et al., 2004), which is used in the ENSEMBL genome annotation pipeline. GeneWise takes a single protein sequence or a profile HMM build from a family of proteins and compares it directly to genomic DNA, taking into account the known statistical properties of gene structures and the presence of sequencing errors. GeneWise can be thought of finding the best gene structure according to its model under the side constraint that the protein aligns well with the given input protein. With this approach, GeneWise is relatively specific, i.e., the predicted genes are frequently correct or at least overlap a true gene. For example, Birney et al., report in an experiment

with human genes that about 90% of the exons predicted by GeneWise were correct when the input protein sequences had between 85% and 95% identity to the target gene. At the same similarity level, they report that 40% of the exons were predicted correctly by GeneWise, a low sensitivity compared with ab initio gene finders. GeneWise and also the whole ENSEMBL pipeline produce therefore a high-specificity gene prediction at the expense of some sensitivity.

Doing two tasks at the same time – gene structure detection and protein alignment – GeneWise is slow and Slater and Birney developed the faster protein-based gene prediction program Exonerate that can also do other types of alignments (Slater et al., 2005).

Another frequently chosen approach to gene finding based on protein homology is to adapt a gene finder that previously did not use protein homology information, e.g., an ab initio gene finder, and to incorporate this new evidence source into the model. This has been done with the programs GenomeScan, AUGUSTUS, and SGP-2 (Parra et al., 2003). Each approach uses external alignments of proteins to the genome (e.g., generated by BLAST) and adjusts the model in a way that exons become more likely in regions that match a protein. These approaches do not suffer from the problem of dropping sensitivity when the similarity is too weak, as regions that do not match to a known protein can still be predicted by the base model.

5. Discussion

Significant improvements could be achieved in automatic genome annotation over the last years, especially when incorporating extrinsic evidence or by developing new mathematical models and machine learning techniques. However, the state of the art in computational gene prediction still has to deal with many issues and problems that were known challenges a few years ago. Nontranscript-based methods still frequently split one gene into several predicted genes or join two neighboring genes into one predicted gene (see Fig. 3). Also, alternative splicing and the UTRs cannot be predicted reliably unless supported by transcript alignments. The accuracy of state-of-the-art systems will most likely improve only gradually unless new insight into the splicing mechanism on the sequence level is achieved.

Currently, the best strategy for annotating a new eukaryotic genome is to combine different gene prediction approaches. Genes that are detectable homologs of known protein families should be identified with protein-based methods and if possible corrected and extended by native cDNA data. Ideally, sufficiently many ESTs should be sequenced to partially support gene structure prediction and to provide training data. Further, when other genomes at an appropriate evolutionary distance exist, the information given by the sequence conservation should be exploited by comparative approaches fine-tuned for the given combination of genomes. Genes or parts of genes in regions with no extrinsic evidence will have to be predicted with ab initio methods trained specifically for the organism. A whole

genome annotation pipeline should either incorporate all the above information directly or several different specialized programs should be run and the results be combined by a combiner program that builds a consensus between several gene prediction sets and extrinsic evidence.

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DEVELOPMENTS IN BIOTECHNOLOGY OF RED ALGAE

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1. Introduction

The marine macroalgae represent one of the components of coastal ecosystem and are of fundamental ecological importance as primary producers in the coastal regions of ocean waters. Of the total marine macrophytic algae, red algae with most ancient eukaryotic lineage are diverse in their habitats and cellular organizations, and comprise the highest number of species diversity over 6,000 followed by brown algae and green algae with 1,700 and 1,200 species, respectively (Guiry and Guiry, 2009). Red algae constitute the largest number of commercially valuable species of the three macroalgal groups, and include species cultivated and harvested as a source of food, phycocolloids, phycosupplements (soil additives, fertilizers, and animal feed), pharmaceuticals, nutraceuticals, and cosmetics (Hanisak, 1998; McHugh, 2003). The most valued of all marine macroalgae is the edible red alga *Porphyra* (or nori in Japanese), which is consumed as food throughout the world and is the basis for the development of farming in Japan, Korea, and China. Total production volume of *Porphyra* worldwide is 900,000 wet tons per annum with a value of US$1.5 billion (Hanisak, 1998; Chopin and Bastarache, 2004). The other important utilization of red seaweeds is as a source of raw material for production of phycocolloids; the gelling, thickening, emulsifying, binding, stabilizing, clarifying, and protecting agents known as carrageenans and agars. *Kappaphycus, Eucheuma*, and *Chondrus* are the principal sources of raw material for production of carrageenan and cultivated extensively in several tropical countries as an alternative livelihood opportunity. Current carrageenan industry worldwide utilizes around 140,000 dry tons harvested through aquaculture practices and produces around 50,000 tons carrageenan with a market value over US$ 600 million (Guiry, 2008). *Gelidium* and *Gracilaria* are the two important agarophytes that form the major part of total agarophytes consumed by world agar industry. Annually, about 125,000 wet tons of agarophytes are processed and 7,500 tons agar produced with a value of US$132 million (Hanisak, 1998; Chopin and Bastarache, 2004).

The most emerging and promising component of seaweed-based industry is the phycosupplement, which is estimated at 1.22 million wet tons and valued at US$53 million (Hanisak, 1998; Chopin and Bastarache, 2004). Most of the tonnage

is used for the manufacturing of soil additives, fertilizers (agrichemicals), and animal feeds. In fact, the Canadian-based private company Acadian Seaplants Limited (ASL), the only large commercial seaweed grower outside of Asia, is a leading exporter of phycosupplement products and accounts for 30–40% of total world market. Further, it is also a world leader in the development of a land-based seawater tank cultivation system involving several seaweed strains. Some of these cultivated seaweeds with edible application have recently found exclusive niche market in Japan providing much higher added value for the crop than those cultured earlier for the purpose of carrageenan. Similarly, the central salt and Marine Chemicals Research Institute (CSMCRI), India, has developed and patented an integrated technology for production of sap (80% fresh wt) from red alga *Kappaphycus alvarezii* along with a residue that yields κ-carrageenan (Eswaran et al., 2005). The sap has turned out to be a promising liquid fertilizer with all essential plant nutrients. Given that the fertilizer will be required in large volumes, there could be a demand for producing very large quantities of biomass in India.

It is evident from the above that global seaweed industry today consumes large volumes of raw material that are largely met from the intensive farming practices. Therefore, the initial studies, in general, on cultivation have largely focused on domestication and selection of strains from wild stocks to multiple the production potentials of cultivated species (Santelices, 1992). Further, the outbreak of diseases like red rot and chytrid blight in *Porphyra* (Fujita, 1990; Park et al., 2006) and ice-ice and epiphytic infection in *Kappaphycus* (Largo et al., 1995a, b; Vairappan et al., 2008) have also been reported to impact the crop yields of farmed red algae causing severe economical losses to the farming industry. As in agriculture, the need to produce better strains for cultivation has been the main impetus behind the biotechnological developments in the red algae. That is, techniques like tissue culture, mutagenesis, and selection developed in the 1980s and then protoplast fusion in the 1990s because classical breeding methods were of little benefit in red macroalgal cultivation. The earlier studies pursued to develop the field of cellular biotechnology of seaweeds have truly succeeded and resulted in establishment of pioneering methods for routine tissue and protoplast culture from a wide range of seaweeds including red algae (see reviews by Polne-Fuller, 1988; Butler and Evans, 1990; Garcia-Reina et al., 1991; Reddy et al., 1994; Aguirre-Lipperheide et al., 1995; Reddy et al., 2008a; Baweja et al., 2009). The advances made in cellular biotechnology have provided the spurt in the studies on molecular biotechnology of seaweeds (see reviews by Stevens and Purton, 1997; Minocha, 2003; Walker et al., 2005; Chan et al., 2006; Reddy et al., 2008b) to realize the benefits offered by seaweed resources to the fullest extent. The previous reports have dealt with either cellular or molecular biotechnology of seaweeds and rarely covering both. The objective of the present report is to review the progress made and discuss the current status of application of cellular and molecular biotechnology techniques exclusively for red seaweeds.

2. An Overview of Cellular Biotechnology and Their Applications

The cellular biotechnology mainly comprises the tissue, callus, and protoplast culture and offers vast opportunities in the area of plant biotechnology, and allowed widespread use of cell culture for *in vitro* genetic manipulation, plant propagation, and production of commercially valuable metabolites. The progress achieved in respective areas is briefly reviewed in the following sections.

2.1. STATUS OF TISSUE AND CALLUS CULTURE IN RED ALGAE

The commercial farming of seaweeds especially agarophytes and carrageenophytes traditionally made use of vegetative thalli for their propagation in the sea (Dublin, 2005; Hurtado et al., 2006; Molloy, 2006; Ganesan et al., 2006, 2009). The practice of selection of seed stock of fastest growing plants of one season as the seed for the next season in *Kappaphycus* and *Eucheuma* led to decline in the crop production (Dawes et al., 1993), which had been attributed to the use of reduced genetic diversity of seed stock. Consequently, several researchers including Cheney (1999) argued that new varieties would have to be produced and preserved using tissue culture and genetic manipulation techniques, which not only increase the crop production rates but also provide a variety of benefits with consequential implications in overall seaweed biotechnology.

There are 51 species belonging to 26 genera that have been successfully grown in tissue culture, including virtually every major commercially valuable species (Table 1). The callus induction rates for red algae varied greatly from species to species. A number of tissue culture studies dealt with agarophytes and carrageenophytes have demonstrated the production of viable micropropagules with a promising application of micropropagation for mariculture (Dawes and Koch, 1991; Reddy et al., 2003; Rajakrishna Kumar et al., 2004, 2007). Later, a technique producing substantially high yields of micropropagules (Fig. 1) from pigmented filamentous callus has been accomplished for commercially important red alga *Kappaphycus alvarezii* (Reddy et al., 2003). Another important and emerging development in this area of research has been the establishment of *in vitro* culture system and photo bioreactors specifically suitable for bioprocess technology for production of useful secondary metabolites with potential medicinal applications (e.g., halogenated monoterpenes, agardhilactone) from select multicellular marine macroalgae under optimized culture conditions (Malaikal et al., 2001; Rorrer and Cheney, 2004). To date, there are only two published reports where tissue culture progeny has been transplanted in the sea and studied for growth. Incidentally, both studies have dealt with farmed *Kappaphycus* and reported daily growth rates of 5–5.5% over 85 days cultivation period in one case (Dawes et al., 1993) and 1.5–1.8 times over the farmed plants propagated through vegetative means in another case (Reddy et al., 2003).

Table 1. List of red algae for which tissue and callus culture accomplished.

Species	PGRs	Medium	Callus induction (%)	Status	Reference
Agardhiella subulata	NAA	mASP12-NTA	NR	PR	Cheney et al. (1987)
	PAA, NAA, BAP, K, 2,4,5 T, IAA	f/5	NR	PR	Bradley and Cheney (1990) and Huang et al. (1998)
Ahnfeltiopsis flabelliformis	IAA, BAP	ASP12-NTA	38	PR	Huang and Fujita (1997)
Carpopeltis affinis	IAA, BAP	ASP12-NTA	96	CI & PR	Huang and Fujita (1997)
C. prolifera	IAA, BAP	ASP12-NTA	60	CI & PR	Huang and Fujita (1997)
Ceramium kondoi	NR	MS	2	CI	Gusev et al. (1987)
Chondrus crispus	K, NAA	SWMD1	NR	PI	Chen and Taylor (1978) and Chen (1982)
Chondracanthus tenellus	IAA, BAP	ASP12-NTA	18	CI & PR	Huang and Fujita (1997)
Kappaphycus alvarezii	NR	PES	10	PR	Polne-Fuller and Gibor (1987), Dawes and Koch (1991) and Dawes et al. (1993)
	NAA, BAP, IAA, K	ESS	90	SE	Reddy et al. (2003)
	NAA, BAP, K, Spm	PES	100	CI	Munoz et al. (2006)
	2-4D, BAP, IAA, Colchicine	VS 50, F/2 50, ASP12-NTA	100	CI & PR	Hayashi et al. (2008)
	PAA, Zea	ESS/2	NR	CI & PR	Hurtado and Biter (2007)
Eucheuma denticulatum	PAA, IBA, IAA, NAA, K, 2ipBAP, Zea	ESS	NR	PR	Dawes and Koch (1991) and Dawes et al. (1993)
E. denticulatum	PAA, Zea	ESS/2	NR	PR	Hurtado and Cheney (2003)
E. uncinatum	NR	PES	3	PR	Polne-Fuller and Gibor (1987)
Furcellaria fastigata	NR	MS	16	CI	Gusev et al. (1987)
Gelidium robustum	NR	PES	2	PR	Polne-Fuller and Gibor (1987)
Gelidiella acerosa	NAA, BAP, IAA, K	PES	93	PR	Rajakrishna Kumar et al. (2004)
G. vagum	NR	MS	15	CI	Gusev et al. (1987)
Gigartina exasperata	NR	PES	3	PR	Polne-Fuller and Gibor (1987)
Gloiopeltis tenax	IAA, BAP	ASP12-NTA	10	CI	Huang and Fujita (1997)
Gracilaria chilensis	IAA, K	PES	NR	CI	Collantes et al. (2004)
G. corticata	NAA, BAP, IAA, K	PES	60	CI & PR	Rajakrishna Kumar et al. (2007)
G. papenfussii	NR	ASP12-NTA	2	PR	Polne-Fuller and Gibor (1987)

G. perplexa	K, IAA, 2-4D	ASP12-NTA	100	CI	Yokoya et al. (2004)
G. tenuistipitata	K, IAA, 2-4D	ASP12-NTA	100	CI	Yokoya et al. (2004)
G. textori	IAA, BAP	ASP12-NTA	5	CI	Huang and Fujita (1997)
G. vermiculophylla	IAA, BAP, 2-4D	ASP12-NTA	NR	CI & PR	Yokoya et al. (1999)
G. verrucosa	NR	M&S	4	CI& PR	Gusev et al. (1987)
	NR	ASP6-F2	30	CI & PR	Kaczyna and Megnet (1993)
Gracilariopsis tenuifrons	IAA, 2-4D, BAP	ASP12-NTA	NR	PR	Yokoya (2000)
Grateloupia acuminata	IAA, BAP	ASP12-NTA	92	CI	Huang and Fujita (1997)
G. doryphora	NR	mPES	100	BF	Robaina et al. (1990)
G. dichotoma	NR	ASP12-NTA		CI	Yokoya and Handro (1996)
G. filiformis	NR	Von stosch	NR	CI&PR	Yokoya et al. (1993)
G. filicina	IAA, BAP	ASP12-NTA		CI	Huang and Fujita (1997)
G. turuturu	IAA, BAP	ASP12-NTA	36	CI	Huang and Fujita (1997)
G. imbricata	IAA, BAP	ASP12-NTA	57	CI	Huang and Fujita (1997)
Hypnea musciformis	NAA, BAP, IAA, K	PES	90	CI	Rajakrishna Kumar et al. (2007)
	IAA, 2-4D, K	ASP12-NTA	NR	CI	Yokoya et al. (2003)
Laurencia sp.	K, BAP, 2-4D, NAA	PES	18	PR	Garcia-Reina et al. (1988)
	NR	PES	40	SR	Robaina et al. (1992)
L. paniculata	K, BAP, NAA, 2-4D	mMS	NR	CI	Gusev et al. (1987)
L. undulata	IAA, BAP	ASP12-NTA		CI	Huang and Fujita (1997)
Meristotheca papulosa	IAA, BAP	ASP12-NTA	2	CI	Huang and Fujita (1997)
Ochtodes secundriramea	Zea, PAA	ASP12-NTA	NR	PR	Malaikal et al. (2001) and Rorrer and Cheney (2004)
Phyllophora nervosa	NR	MS	18	CI&PR	Gusev et al. (1987)
Porphyra perforata	NR	ASPC-1 & ASP12-NTA	89	PR	Polne-Fuller and Gibor (1987)
P. lenceolata	NR	ASP6-F2	84	PR	Polne-Fuller and Gibor (1987)
P. nereocystis	NR	ASP12-NTA	90	PR	Polne-Fuller and Gibor (1987)
P. umbilicalis	NAA, K	La	80	CI	Liu and Kloareg (1992)
P. yezoensis	NR	NR		PR	Yamazaki et al. (1998) and Hafting (1999)

(continued)

Table 1. (continued)

Species	PGRs	Medium	Callus induction (%)	Status	Reference
Prionitis crispata	IAA, BAP	ASP12-NTA	65	CI	Huang and Fujita (1997)
Pterocladia capillacea	NAA, K	mPES	12	CI	Liu and Gordon (1987)
	NR	NSW, PES, mNSW, mPES		CI&BS	Liu et al. (1990)
Ptilophora subcostata	IAA, BAP	ASP12-NTA	39	CI	Huang and Fujita (1997)
Rhodymenia pertusa	K, BAP, NAA, 2-4D	mMS	NR	CI	Gusev et al. (1987)
Smithora naiadum	NR	ASPC1	85	PR	Polne-Fuller and Gibor (1987)
Solieria filiformis	NR	$NaNO_3$ + NaH_2PO_4	90.3	CI	Robeldo and Garcia-Reina (1993)

ASP6-F2, artificial seawater medium (Fries); ASP-12NTA, artificial seawater medium with nitrlotriacetic acid; PES, provosoli enriched seawater medium; ESS, erdshcribers seawater; MS, Murashige and Skoog; BAP, benzylaminopurine; 2-4D, 2,4,dichlorophenoxy acetic acid; IAA, indole acetic acid; K, kinetin; NAA, naphthalene acetic acid; PAA, phenyl acetic acid; 2,4,5 T, IAA, 2,4,5 trichlorophenoxy acetic acid; Zea: zeatin; Spm, spermine; NR, not reported; BF, bud formation; CI, callus induction; PR, plant regeneration; SE, somatic embryogenesis; AV, adventitve embryogenesis; BS, biochemical study; SD, shoot differentiation.

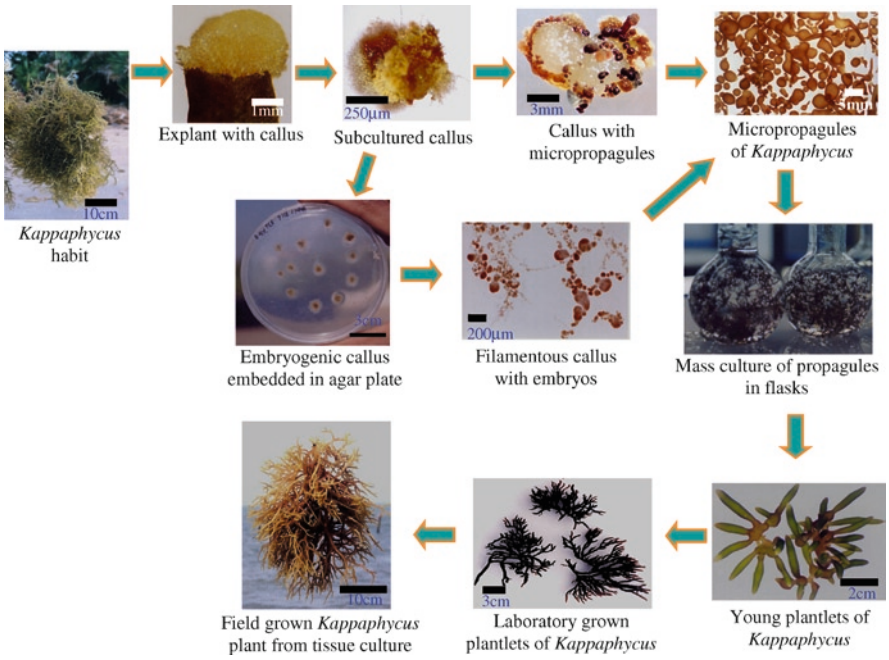

Figure 1. Mass production of micropropagules from pigmented filamentous callus of *Kappaphycus alvarezii*.

The role of plant growth regulators (PGRs) on callus induction and differentiation in multicellular algae is often reported with divergent views (Bradley, 1991; Evans and Trewavas, 1991). Nevertheless, indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin had stimulatory role in callus formation, growth, and regeneration both in intercalary and apical explants of *Gracilaria tenuistipitata*, *G. perplexa*, and *Grateloupia dichotoma* (Yokoya and Handro, 1996; Yokoya et al., 2004). Additions of a combination of auxins and cytokinins to culture medium resulted in induction of callus in *Gracilaria verrucosa* (Kaczyna and Megnet, 1993). The implications of PGRs on callus formation have also been reported to vary with the seaweed and photon flux densities used during explant culture. The brown color morph of *Hypnea musciformis* had the highest rate of callus formation in high photon irradiance with low concentration of IAA, while green color morphs produced calli with 2,4-D irrespective of concentration and photon flux densities used. Similarly, IAA:BA (5:1 mg l^{-1}) stimulated callus formation in *Kappaphycus* strain originated from tetraspore germination. Treatment of explants with colchicin (0.01%) for 14 days in half strength von Stosch's medium with glycerol (90 mM) further increased the regeneration potential of callus (Hayashi et al., 2008). A water-soluble extract from *Laurencia* sp. increased callus

formation on explants of the same species (Robaina et al., 1992). In view of these conflicting findings, it would be interesting to test the use of some PGR conjugates that have been recently reported in a number of seaweeds (Stirk et al., 2005).

Cell suspension cultures for red algae have not been so far achieved except for *Porphyra* (Chen, 1989). The possible reason could be attributable to their callus structure and morphology. In most cases, the callus from macroalgae is composed of uniseriate branched filaments and is rigid (nonfriable). In contrast to friable callus of higher plants, the filamentous callus regenerate directly into full plants via microplantlet stage when transferred to agitated liquid cultures. The bioprocess engineering studies carried out in *Agardhiella subulata* (Huang et al., 1998), *Ochtodes secundiramea* (Malaikal et al., 2001), and *Portieria hornemannii* (Barahona and Rorrer, 2003) have utilized the microplantlets and filamentous clumps obtained from cell and tissue culture for continuous culture system. While these suspension culture systems may not look like a typical cell culture system of a land plant, they are functionally analogous to cell suspension cultures of higher plants and differ in many ways from whole plants. They typically consist of a mass of tiny shoots, which can be subcultured and grown in photo-bioreactors in the same fashion as a cell suspension culture.

2.2. TRENDS IN PROTOPLAST RESEARCH OF RED ALGAE

Protoplasts form another important component of cellular biotechnology. Protoplast isolation, regeneration, and somatic hybridization through protoplast fusion are of particular interest in seaweed biotechnology and genetic improvement of cultivars. Protoplasts are living plant cells devoid of rigid cell wall and with their totipotent potential and homogeneous cell system facilitate the exploration of several aspects of modern biotechnology. Treatment of cells or tissues with specific cell wall degrading enzymes under defined conditions results in total removal of their complex polysaccharide cell wall. Although the first protoplasts produced from marine macroalgae were obtained using mechanical methods (e.g., Tatewaki and Nagata, 1970; Enomoto and Hirose, 1972), it was not until the discovery of suitable enzymes for cell wall digestion for the field to gain momentum in late 1980s and early 1990s. Soon after, protoplasts had been isolated from over 40 species (Table 2) of different red algae. The two important genera that undoubtedly received overwhelming attention for development of protoplast studies are *Gracilaria* and *Porphyra* because of their proven commercial importance in food and biotechnology industry.

The totipotency property of algal protoplasts has allowed their consideration for a variety of applications in both fundamental and applied research. The protoplasts of *Porphyra* have been used for establishing the select cell lines having resistance to low salinity (Iwabuchi, 1995), higher temperatures (Masuda et al., 1995), and with altered amino acid and amino acid analog contents (Yamashita and Fujita, 1996). In another study, protoplasts from *Porphyra* have also been successfully

Table 2. Red algal species for which protoplast production reported.

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
Acrosorium polyneurum	Cellulase R-10	4%		PI	Yamaguchi et al. (1989)
	Macerozyme	2%			
	Agarase	50 U			
	Papain	2%			
	Mannitol	0.6 M			
Bangia atropurpurea	Pretreatment		5.7×10^6	PI	Araki et al. (1994)
	Papain	2%			
	Mannitol	0.5 M			
	MES	20 mM			
	Treatment				
	Agarase	1 U			
	β-1,4-Mannanase	1 U			
	Xylanase	1 U			
	Mannitol	0.7 M			
	MES	20 mM			
	pH	6.0			
Chondrus crispus	Carragenase	1%		BS	Smith and Bidwell (1989)
	Cellulase	1%			
	Pectolyase	1%			
	Macerozyme	0.5%			
	Sorbitol	14.5%			
	Sea Salt	2.4%			
	pH	7.5			
	Pretreatment		5.8×10^8	PI	Le Gall et al. (1990)
	NaCl	450 mM			
	$MgCl_2$	120 mM			
	Tris	100 mM			
	PMSF	0.2 mM			
	Treatment				
	Cellulase	1%			
	Carragenase	180 U			
	Sorbitol	0.35 M			
	NaCl	0.5 M			
	$MgCl_2$	40 mM			
	PMSF	0.2 mM			
	KCl	5 mM			
	Tris	50 mM			
	pH	6.0			
Gelidium robustum			$1–8.5 \times 10^8$	PI	Coury et al. (1993)
Gracialria asiatica	Pretreatment			PI	Yan and Wang (1993)
	SW:DW	60:40			
	Mannitol	1 M			
	Treatment				
	Crude Agarase				
	Cellulase	4%			
	Sea snail enzyme	1%			
	SW:DW	60:40			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	Mannitol	0.8 M			
	$CaCl_2$	5 mm			
	pH	6.2–6.5			
G. changii	Pretreatment		5–8 × 10^4	PR	Yeong et al. (2007)
	SW:DW	60:40			
	Mannitol	1.0 M			
	Treatment				
	Cellulase	2%			
	Macerozyme	1%			
	Agarase	10 U/ml			
	MES	50			
	pH	6.0			
G. chilensis	NA	NA	NA	PR	Cheney (1990)
G. chorda	Cellulase	3%	0.2–20 × 10^5	PI	Chou and Lu (1989)
	Macerozyme	3%			
	Pectolyase	0.5%			
	Abalone acetone powder	10%			
	Mannitol	0.8 M			
	$CaCl_2$	5 mM			
	pH	5.8–6.0			
G. filicina	Pretreatment		10 × 10^6	PI	Yamaguchi et al. (1989)
	Papain	2%			
	NaCl	2%			
	Mannitol	0.7 M			
	Tris	50 mM			
	pH	7.5			
	Treatment				
	Bacterial crude extract				
	Cellulase	0.5%			
	Macerozyme	0.2%			
	Top shell powder	2.5%			
	MES	25 mM			
	pH	6.0			
G. gigas	Cellulase	3%	0.2–20 × 10^5	PI	Chou and Lu (1989)
	Macerozyme	3%			
	Pectolyase	0.5%			
	Abalone acetone powder	10%			
	Mannitol	0.8 M			
	$CaCl_2$	5 mM			
	pH	5.8–6.0			
G. lemaneiformis	Cellulase R-10	3%	3–10 × 10^5	CW	Cheney et al. (1986)
	Macarozyme	3%			
	Agarase	1%			
	Pectolyase	0.5%			
	Mannitol	1 M			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	$CaCl_2$	5 mM			
	SW:DW	60:40			
	MES	50 mM			
	pH	6.0			
G. lemaneiformis	Cellulase	2%	10^5× 10^7	PI	Bjork et al. (1990)
	Agarase	0.01%			
	Mannitol	0.4 M			
	pH	7.0			
G. salicornia	Cellulase	3%	0.2–20 × 10^5	PI	Chou and
	Macerozyme	3%			Lu (1989)
	Pectolyase	0.5%			
	Abalone powder	10%			
	Mannitol	0.8 M			
	$CaCl_2$	5 mM			
	pH	5.8–6.0			
G. sordida	Cellulase	2%	10^5–10^7	PI	Bjork et al. (1990)
	Agarase	0.01%			
	Mannitol	0.4 M			
	pH	7.0			
G. tenuistipitata	Cellulase	3%	0.2–20 × 10^5	PI	Chou and Lu (1989)
	Macerozyme	3%			Bjork et al. (1990)
	Pectolyase	0.5%			
	Abalone powder	10%			
	Mannitol	0.8 M			
	$CaCl_2$	5 mM			
	pH	5.8–6.0			
G. tikvahiae	Cellulase R-10	3%	3–10 ×10^5	PR	Cheney et al. (1986)
	Macerozyme	3%			Cheney (1990)
	Agarase	1%			
	Pectolyase	0.5%			
	Mannitol	1 M			
	$CaCl_2$	5 mM			
	SW:DW	60:40			
	MES	50 mM			
	pH	6.0			
G. verrucosa	Cellulase	2%	10^5–10^7	PI	Bjork et al. (1990)
	Agarase	0.01%			Araki et al. (1998)
	Mannitol	0.4 M			
	pH	7.0			
G. verrucosa	Pretreatment		8–10 × 10^6	CW	Mollet et al. (1995)
	CaCl2.2H2O	10 mM			
	Mannitol	0.4 M			
	Tris MES	10 mM			
	pH	6.0			
	Treatment				
	Agarase	25 U			
	$CaCl_2 \cdot 2H_2O$	10 mM			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	Cellulase	1.25%			
	Mannitol	0.8 mM			
	Tris Mes	10 mM			
	pH	6.0			
Grateloupia sparsa	Cellulase	4%	7×10^7–7×10^8	CW	Chen and Chiang (1995)
	Macerozyme	2%			
	Agarase	50 U			
	Papain	2%			
	Mannitol	0.6 M			
G. filicina	Pretreatment		10×10^6	PI	Yamaguchi et al. (1989) Chen and Chiang (1994)
	Papain	2%			
	NaCl	2%			
	Mannitol	0.7 M			
	Tris	50 mM			
	pH	7.5			
	Treatment				
	Bacterial extract				
	Cellulase	0.5%			
	Macerozyme R-10	0.2%			
	Top shell powder	2.5%			
	MES	25 mM			
	pH	6.0			
G. turuturu	Pretreatment		10×10^6	PI	Yamaguchi et al. (1989)
	Papain	2%			
	NaCl	2%			
	Mannitol	0.7 M			
	Tris	50 mM			
	pH	7.5			
	Treatment				
	Bacterial crude extract				
	Cellulase	0.5%			
	Macerozyme R-10	0.2%			
	Top shell powder	2.5%			
	MES	25 mM			
	pH	6.0			
Halymenia formosa	Cellulase	5%	2×10^4	PI	Chou and Lu (1989)
	Abalone acetone powder	6.7%			
	Mannitol	1 M			
	pH	6.0			
Kappaphycus alvarezii	Crude cellulase	1%	$1–1.2 \times 10^7$	PI	Zablackis et al. (1993)
	Pure k carragenase	195 U			
	κ & ι carragenase	108 U			
	Sorbitol (Os/kg)	1.5			
	Abalone powder	5%	8.2×10^3	PR	Salvador and Serrano (2005)
	Cellulase	2%			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	Mannitol	1.0 M			
	CaCl2	5 mM			
	pH	6.0–6.1			
Laurencia obtusa	Sea urchin powder	10%		PI	Balestri et al. (1989)
	Sorbitol	0.8M			
	CaCl2	3.4 mM			
	Phosphate buffer	0.1 M			
	pH	6.0			
Palmaria palmata	Cellulase	3%	$4–6 \times 10^7$	PI	Liu and Kloareg (1992)
	Abalone powder	0.1–0.2%			Nikolaeva
	PES	60%			et al. (1999)
	Phosphate buffer	40%			
	Mannitol	0.1 M			
	CaCl2	5 mM			
	pH	6.0–6.2			
Palmaria palmata	Cellulase R-10	2%	5×10^7	PS	Le Gall et al. (2004)
	Macerozyme R-10	1%			
	Abalone enzyme	2%			
	Bovine albumin	0.5%			
	$Na_3C_6H_5O_7.2H_2O$	50 mM			
	Mannitol	0.8 M			
	pH	5.8			
Plocamium cartilagineum	Sea urchin powder	10%		PI	Balestri et al. (1989)
	Sorbitol	0.8 M			
	$CaCl_2$	3.4 mM			
	Phosphate buffer				
	pH	6.0			
Porphyra.sp	Crude enzyme	187.5 µL	500/field	PI	Packer (1994)
	Paua gut extract	37.5 µL			
	Aspergillus extract	125 µL			
P. angusta	Cellulase	5%	2×10^5	PI	Chou and Lu (1989)
	Abalone powder	6.7%			
	Mannitolo	1 M			
	pH	6.0			
P. crispata	Cellulase	5%	2×10^5	PI	Chou and Lu (1989)
	Abalone powder	6.7%			Gall et al. (1993)
	Mannitol	1 M			
	pH	6.0			
P. dentata				PI	Gall et al. (1993)
P. lanceolata	Abalone powder	10%		PR	Polne-Fuller
	Sorbitol	0.6 M			et al. (1984)
	HEPES	50 mM			
	pH	6.0			
P. leucosticta	Sea snail	2%		PR	Chen (1987)
	Cellulase	1.5%			
	Pectolyase	0.5%			
	Sorbitol	0.5 M			
	$CaCl_2$	0.08%			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	NaH_2PO_4	0.01%			
	$Ca(H_2PO_4)H_2O$	0.01%			
	pH	7.0			
P. linearis	Sea snail extract	2%		PR	Chen et al. (1988)
	Cellulase R-10	3%			Chen (1989)
	Sorbitol	0.5 M			
	pH	7.0			
P. linearis	Agarase	0.2%		PR	Chen et al. (1995)
	Mannitol	0.1 M			
	pH	6.8			
P. nereocystis	Pretreatment		$12–18 \times 10^6$	PR	Waaland et al. (1990)
	Papain	10%			
	Mannitol	0.5 M			
	MES	50 mM			
	pH	6.0			
	Treatment				
	Abalone powder	2%			
	Mannitol	0.5 M			
	MES	50 mM			
	pH	6.0			
P. okamurae	Pretreatment		5×10^5	PI	Fujita and
	Proteolyase	5%	1.5×10^7		Saito (1990)
	Mannitol	0.6 M			
	HEPES	50 mM			
	pH	8.0			
	Treatment				
	Bacterial extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. okhaensis	Protease P6	1%	$23.2 \pm 0.24 \times 10^6$	PI	Dippakore et al. (2005)
	Cellulase	2%			
	Macerozyme R-10	2%			
	Agarase	50 U			
	Abalone powder	1%			
	Dextran sulfate	0.5%			
	NaCl	1%			
	HEPES	25 mM			
	pH	6.0			
P. perforata	Abalone acetone powder	10%		PR	Polne-Fuller et al. (1984, 1990)
	Sorbitol	0.6M			Saga et al. (1986)
	HEPES	50 mM			
	pH	6.0			
P. pseudolinearis	Pretreatment		$5 \times 10^5–1.5 \times 10^7$	PR	Fujita and Saito (1990)
	Proteolyase	5%			
	Mannitol	0.6 M			
	HEPES	50 mM			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	pH	8.0			
	Treatment				
	Bacterial extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. seriata	Pretreatment		5×10^5–1.5×10^7	PI	Fujita and Saito (1990)
	Proteolyase	5%			
	Mannitol	0.6 M			
	HEPES	50 mM			
	pH	8.0			
	Treatment				
	Bacterial crude extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. suborbiculata	Limpets extract			CW	Tang (1982)
	Cellulase	2%			
	Glucose	2.0 M			
P. suborbiculata	Pretreatment		5×10^5–1.5×10^7	PI	Fujita and Saito (1990)
	Proteolyase	5%			
	Mannitol	0.6 M			
	HEPES	50 mM			
	pH	8.0			
	Treatment				
	Bacterial crude extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. tenera	Pretreatment		2.5×10^6	PI	Song Ho and Chung (1988)
	Papain	2.5%			
	Mannitol	0.7M			
	Tris	50 mM			
	pH	7.5			
	Treatment				
	Abalone acetone powder				
	Mannitol	0.7 M			
	$CaCl_2$	5 mM			
	NaCl	2%			
	MES	25 mM			
	pH	7.5			
	Pretreatment		5×10^5–1.5×10^7	PI	Fujita and Saito (1990)
	Protease	5%			
	Mannitol	0.6 M			
	HEPES	50 mM			

(continued)

Table 2. (continued)

Species	Enzyme composition		Yield (g^{-1} f wt.)	Status	Reference
	pH	8.0			
	Treatment				
	Bacterial crude extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. tenuipedalis	Pretreatment		5×10^5–1.5×10^7	PR	Fujita and Saito (1990)
	Protease	5%			Saga and Sakai (1984)
	Mannitol	0.6 M			Fujita and Migita (1985)
	HEPES	50 mM			
	pH	8.0			
	Treatment				
	Bacterial crude extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
P. yezoensis	Pretreatment		8.6×10^5	PR	Araki et al. (1987)
	Papain	2%			Yamaguchi et al. (1989)
	Mannitol	0.7 M			
	MES	50 mM			
	Treatment				
	β-1,4-Mannanase	1 U			
	β-1,4-xylanase	1 U			
	pH	6.0			
P. yezoensis	Pretreatment		5×10^5–1.5×10^7	PI	Fujita and Saito (1990)
	Protease	5%			
	Mannitol	0.6 M			
	HEPES	50 mM			
	pH	8.0			
	Treatment				
	Bacterial extract				
	Dextran sulfate	0.5%,			
	HEPES	50 mM			
	pH	8.0			
	β-Mannanase,	5U		PI	Ootsukaa et al. (2006)
	Mannitol	0.7 M			
Solieira filiformis	Cellulysin	3%	8.7×10^5	PI	Gomez-Pinchetti and Garcia Reina (1993)
	Cellulase	3%			
	Abalone powder	3%			
	Agarase	0.01%			
	Pectolyase	0.25%			
	Carragenase	30 U			
	Mannitol	0.4 M			
	pH	6.0			

BS, biochemical study; CW, cell wall formation; PI, protoplast isolation; PR, plant regeneration; GS, genetic study; NA, not accessible; SW, seawater; DW, distilled water.

tested for their seeding and regeneration in laboratory conditions (Dippakore et al., 2005). There also exist a number of examples where protoplasts have been used initially for investigating the basic physiological and biochemical mechanisms. The inorganic carbon uptake and assimilation mechanisms in *Chondrus crispus* (Smith and Bidwell, 1989) and *Gracilaria tenuistipitata* (Haglund et al., 1992) have been investigated using photosynthetically active protoplasts. A number of studies have also confirmed that the physiological state of protoplasts is as same as that of intact plants (Davison and Polne-Fuller, 1990; Amano and Noda, 1992). Secretion of carrageenan fragments by cultured protoplasts of *Kappaphycus alvarezii* var. *tambalang* has also been reported (Zablackis et al., 1993).

In the past, most seaweed strain improvement efforts have been confined to the use of classical breeding techniques (strain selection, mutagenesis, and sexual hybridization). Such efforts have met with little success in producing commercially valuable strains in red algae. However, the major barrier in classical breeding techniques, especially sexual hybridization, is obtaining both sexes of both species at a time. For example, in *Kappaphycus* and *Gelidiella*, male plants are rare and in some species unknown. Further, the red algae appear to lack interspecific interfertility. In contrast, somatic hybridization by means of protoplast fusion facilitates mixing of two genomes of different origin, which is otherwise not possible due to sexual incompatibility. This technique, in contrary to genetic engineering, provides an important means to transfer the traits particularly polygenetic in nature.

The first report of successful protoplast fusion and fusion product regeneration has been between two color morphs of red alga *Porphyra yezoensis*, which is popularly consumed as human food (Fujita and Migita, 1987). Of the total 21 reports available on the protoplast fusion, 17 are related to commercially important red seaweeds (Table 3) and of which, 11 are of intrageneric fusion and the rest five are of intergeneric fusions (Uppalapati and Fujita, 2000; Uppalapati et al., 2000; Reddy et al., 2008b). The genus *Porphyra*, being an important edible red alga and also susceptible to many marine pathogens, has been the first target for application of these techniques to the genetic improvement of this alga. Fujita and his coworkers have extensively carried out interspecific protoplast fusion between *Porphyra yezoensis* and *P. pseudolinearis* and screened the F1 hybrids for resistance to red rot and chytrid blight. These studies have revealed that some F1 thalli showed relatively low infection levels to chytrid blight than red rot when compared with the parent *P. yezoensis* (Fujita and Uppalapati, 1997). The intergeneric F1 fusion products thalli of *P. yezoensis* and *Bangia atropurpurea* showed very low infection levels to both red rot and chytrid blight in comparison with *P. yezoensis* (Fujita, 1993). The heterokaryons regenerated were mostly characterized at biochemical levels. Later studies with intergeneric protoplast fusion between *Porphyra yezoensis* and *Monostroma nitidum* have employed RAPD markers in addition to biochemical parameters for characterizing the somatic hybrids (Kito et al., 1998). The methods for screening and selection of heterokaryons are underdeveloped for seaweeds. The efficient implementation of screening methodologies using differential fluorescence labeling, and genetic and metabolic complementation can

Table 3. Present status of protoplast fusion and regeneration in different red algae.

Fusion species	Method of fusion	Status	Reference
Enteromorpha × *Porphra yezoensis*	PEG	PF	Saga et al. (1986)
Gracilaria chilensis × *G. tikvahiae*	PEG	PD	Cheney (1990)
P. yezoensis × *P. yezoensis*	PEG	PD	Fujita and Migita (1987)
P. yezoensis × *P. pseudolinearis*	Electrofusion	PD	Fujita and Saito (1990)
P. yezoensis × *P. haitanensis*	PEG	CF	Dai et al. (1993)
P. yezoensis × *P. tenera* (green)	PEG	CF	Araki and Morishita (1990)
P. yezoensis × *P. suborbiculata*	Electrofusion	CF	Mizukami et al. (1995)
P. yezoensis × *P. vietnamensis*	No information	CF	Matsumoto et al. (1995)
P. tenera × *P. suborbiculata*	No information	CF	Matsumoto et al. (1995)
P. linearis × *P. miniata*	Electrofusion	CF	Chen et al. (1995)
P. suborbiculata × *P. tenuipedalis*	Electrofusion	CF	Achiha (1995)
P. yezoensis × *Bangia atropurpurea*	No information	CF	Fujita (1993)
P. pseudolinearis × *B. atropurpurea*	No information	CF	Fujita (1993)
P. yezoensis × *Monostroma nitidum*	PEG	PD	Kito et al. (1998)
P. yezoensis × *P. tenuipedalis*	PEG	PD	Uppalapati and Fujita (2000)
P. yezoensis × *Enteromorpha compressa*	PEG and electrofusion	PF	Uppalapati et al. (2000)
P. yezoensis × *M. nitidum*	PEG and electrofusion	PF	Uppalapati et al. (2000)

PF, protoplast fusion; PD, protoplast development; CF, callus development.

greatly accelerate the success rate. Also, the frequency of cybrids from somatic hybridization can be further enhanced by inactivating the nuclei of either of partner by x-rays, γ-rays, and mutagenic treatments.

To circumvent some of the inherent problems associated with protoplasts like poor survival rates, especially for anatomically more complex red algae, Cheney (1999) used a technique called "spore–protoplast fusion" and reported greater survival rates of hybrids in a number of red macroalgal genera, including *Porphyra*, *Gracilaria*, and *Chondrus*.

3. Molecular Biotechnology of Red Algae

The genetic engineering of red seaweeds is aimed at the genetic improvement of commercially important species for the production of gel forming polysaccharides, improved nutritional quality and growth rates, the production of fine chemicals (medicines and pigments), the production of disease-resistant varieties, and for their potential use in phyto-remediation of aquatic environments. The advances made in molecular approaches have led to better understanding of the red algal systematics, phylogeny, and their evolutionary relationships. The red algal population studies using molecular markers have also been briefly discussed in the context of molecular biotechnology.

3.1. STATUS OF GENETIC ENGINEERING

The genetic engineering studies carried out in macroalgae so far have mainly dealt with commercially valuable red and brown seaweeds only. The rate and efficiency of gene expression delimits the progress of studies in seaweeds when compared with unicellular algae (Fukuda et al., 2008). Table 4 shows the status of genetic engineering studies accomplished for red algal taxa. The early studies have demonstrated the transient expression of the β-glucoridinase reporter gene (*uidA*) fused to the CaMV-35S promoter in a number of macroalgal taxa using various gene transfer methods including biolistic particle bombardment, vector mediated, and electroporation (Kurtzman and Cheney, 1991; Kubler et al., 1994; Kuang et al., 1998). In another study, Gan et al. (2003) reported the transient expression of reporter gene *lacZ* fused to the SV40 promoter in *Gracilaria changii*. The subsequent studies attempted to use homologous promoters for foreign gene expression in *Porphyra* protoplasts using a portion of 18S rDNA in a vector pQD-GUS, and compared the expression of GUS protein with that of the parent pBS-GUS vector. The resultant transformants showed increased GUS activity with pQD-GUS compared with those of pBS-GUS. Following the *Agrobacterium* mediated gene transfer, Bernasconi et al. (2004) reported transformation of *Porphyra yezoensis* expressing bacterial nitroreductase gene (*nfs1*) with abilities to detoxify trinitrotoluene (TNT). The attempts were also made for efficient transient expression of gene construct with GAPDH promoter of *P. yezoensis* by particle bombardment (Fukuda et al., 2008). Although progress has been made in the genetic engineering of red macroalgae, it is not well developed as that for kelps as evidenced by recent reports by Qin et al. (2005). Some of the reasons for this may have to do with the lack of appropriate promoters and other factors described in Fukuda et al. (2008). The continuing research in genetic engineering will lead to the development of transgenic red algae with desired and improved qualities.

Table 4. Red algal species for which genetic engineering accomplished.

Species	Mode of gene transfer	Status of expression	Reference
Chondrus crispus	Vector mediated	Stable	Collen et al. (2006)
Gracilaria changii	Biolistic particle	Transient	Gan et al. (2003)
Kappaphycus alvarezii	Biolistic particle	Transient	Kurtzman and Cheney (1991)
Porphyra leucosticta	Electroporation	Transient	Lin et al. (2001)
P. miniata	Electroporation	Transient	Kubler et al. (1994)
P. tenuipedalis	Electroporation	Transient	Achiha (1995)
P. yezoensis	Electroporation	Transient	Kuang et al. (1998)
P. yezoensis	Vector mediated	Stable	Cheney (1999)
P. yezoensis	Electroporation	Transient	He et al. (2001)
P. yezoensis	Vector mediated	Stable	Cheney (2001)
P. yezoensis	Vector mediated	Transient	Liu et al. (2003)
P. yezoensis	Electroporation	Transient	Mizukami et al. (2004)

3.2. SEQUENCE ANALYZED MARKERS FOR RED SEAWEED SYSTEMATICS

The red algae with complex alternation of life cycles and great morphological plasticity have necessitated the investigations into their genetic constituents. Furthermore, the mating patterns, gene flow among the individuals during sexual reproduction, and genetic structure of populations are also not well understood for this diverse group of plants. In this context, classical approaches like study of reproductive anatomy (Gargiulo et al., 1992), chemistry (Bird et al., 1987), cytogenetics, and karyology (Plastino and Oliveira, 1988; Bird et al., 1990; Godin et al., 1993) have been employed to understand the taxonomical and evolutionary hierarchy of different red algal taxa. The application of molecular tools and techniques has advanced the resolution of taxonomy and evolutionary concepts about the red seaweeds. The often used molecular markers for studying the systematics and evolutionary relationship of red seaweeds are shown in Table 5. The molecular approaches that were used to study the systematics of algae include the DNA fingerprinting (Rice and Bird, 1990; Wattier et al., 1997) and gene sequence data (Destombe and Douglas, 1991; Bird et al., 1994; Goff et al., 1994). The studies on phylogenetic relatedness of seaweeds have progressed with the generation of sequence data from the conserved organelle markers of nuclear encoded internal transcribe region (Bhattacharya et al., 1990; Goff et al., 1994; Bellorin et al., 2002), plastid-encoded *rbcL* gene and rubisco spacer gene (Wang et al., 2000; Clerck et al., 2005), mitochondrial *cox*2, *cox*3 gene, and *cox* spacer region (Zuccarello et al., 1999; Saunders, 2005; Robba et al., 2006; Yang et al., 2008). The application of multiple markers provided additional opportunity to distinguish the differentiations at genus or species level in *Porphyra* based on plastid-encoded *psa*A, *psb*A gene, and phycoerythrin gene (Yang and Boo, 2004). Recently, partial amplication of intergenic spacer sequence has been reported in *P. haitensis* (Li et al., 2009). The intergenic spacer sequences with high evolution rates than internal transcribed spacer sequences can be employed with greater efficiency for hierarchal distinctness of even closely related species.

3.3. DEVELOPMENTS IN MOLECULAR GENETIC MARKERS

The genetic markers have been developed for the identification of plant variants, the analysis of phylogenetic and genetic diversity, hybrid confirmation, genome mapping, and gene tagging for marker-assisted selection studies. The discriminatory markers were developed using restriction fragment length polymorphism (RFLP) for *Porphyra* (Stiller and Waaland, 1993; Teasdale et al., 2002; Niwa et al., 2006) and others also for the screening of population of *Gracilaria* (Candia et al., 1999). The advancement in the genetic studies was possible only after the development of PCR-based genetic markers due to their simplicity and high reproducibility. Of the PCR-based markers, RAPD markers are quite successful enough to classify even at species level and used in *Gracilaria* (González et al., 1996; Lim et al.,

Table 5. Phylogenetic markers for red algae.

Gene	Primer sequence	Reference
cox 1	5′-TCAACAAATCATAAAGATATTGG-3′ 5′-ACTTCTGGATGTCCAAAAAAYCA-3′	Saunders (2005)
cox 1	5′-CCT GTN TTA GCA GGW GCT ATT ACA ATG C-3′ 5′-ACA GTA TAC ATA TGA TGN GCT CAA AC-3′	Geraldino et al. (2006)
*cox*2-3 spacer	5′-GTACCWTCTTTDRGRRKDAAATGTGATGC-3′ 5′-GGATCTACWAGATGRAAWGGATGTC-3′	Zuccarello et al. (1999)
Nuclear ITS	5′-dTGTACACACCGCCCGTCGC-3′ 5′-dATATGCTTAARTTCAGCGGGT-3′	Bellorin et al. (2002)
rbcl	5′-GGTGAATTCCATACGCTAAA ATG-3′ 5′-GCAGCTGTAACATTCATGTA-3′	Wang et al. (2000)
rbcl	5′-CCCTTTATGCGATGGAAAGA-3′ 5′-GCAGCAGTTACATTCATATA-3′	Wang et al. (2000)
rbcS	5′-TAATAACGAGAACCTTCTGG-3′ 5′-TARTAACGAGARCCYTCWGG-3′	Lee et al. (2001)
Rubisco spacer	5′-TGTGGACCTCTACAAACAGC-3′ 5′-CCCCATAGTTCCCAAT-3′	Maggs et al. (1992)
Rubisco spacer	5′-TATACTTCTACAGACACAGCTGA-3′ 5′-ATTTCACACAGGAAACAGCTATGACATGTCAAA TAAT GGTAGTCCCCA-3′	Zuccarello et al. (1999)
*RPB*1	A Fwd. 5′-GA(G/T)TGTCC(T/G)GG(A/T)CATTTTGG-3′ D Fwd. 5′-TACAATGC (T/C)GA(T/C)TT(T/C)GA(T/C)GG-3′ F Revs. 5′-C(T/A)CG(T/A)CC(T/A)CCCAT(T/A)GC(A/G)TGG-3′ GRevs.5′-TG(A/G)AA(I/C/T)GTITT(I/C/T)AG(I/T)GTCAT(C/T)TG-3′	Stiller and Hall (1997)
*psa*A	psaA130F 5′-AACWACWACTTGGATTTGGAA-3′ psaA180F 5′-GATAGTCAWACHAGTTCWTTAGA-3′ psaA1600R 5′-GCATGAATATGRTGWACCAT-3′ psaA1760R 5′-CCTCTWCCWGGWCCATCRCAWGG-3′	Yoon et al. (2002)
*psb*A	psbA-F 5′-ATGACTGCTACTTTAGAAAGACG-3′ psbA500F 5′-CTCTGATGGWATGCCWYTAGG-3′ psbA600R 5′-CCAAATACACCAGCAACACC-3′ psbA-R2 5′-TCATGCATWACTTCCATACCTA-3′ psbA-R1 5′-GCTAAATCTARWGGGAAGTTGTG-3′	Yoon et al. (2002)

2001), *Gelidium* (Patwary et al., 1993; Bouza et al., 2006), *Porphyra* (Dutcher and Kapraun, 1994; Huh et al., 2006). Even the sex specific RAPD markers have been investigated for *Gracilaria gracilis* and *G. Changii* (Martinez et al., 1999; Sim et al., 2007). Precision of genetic marker studies have further advanced following the development of AFLP markers, which offered greater sensitivity and reproducibility when compared with previously developed DNA markers. The first report of use of AFLP marker was by Donaldson et al. (1998) while studying the population of *Chondrus crispus*. The subsequent studies carried out on population genetics of *Porphyra* (Iitsuka et al., 2002; Niwa et al., 2006) and *Chondrus* (Donaldson et al., 2000) have also employed the AFLP markers. Further refinement in the development of genetic marker studies were made by codominant ISSR markers in

Gracilaria lemaneiformis, Chondrus crispus (Xue et al., 2006; Wanga et al., 2007). In addition to random genetic markers, cleaved analyzed polymorphic sequence (CAPS) of four gene loci, beta-tubulin gene (ß-tubulin), elongation factor-1 gene (EF-1), type II DNA topoisomerase gene (TOP2), and the c-subunit of the vacuolar-type Hþ -ATPase gene (V-ATPase) for studying the genetic diversity within related species of *P. yezoensis* (Park et al., 2007).

3.4. GENOMIC ORGANIZATION OF RED SEAWEED

The last few years have witnessed considerable progress in the field of algal genomics. Recently, complete genome sequences from the unicellular red alga *Cyanidioschyzon merolae* have been published (Matsuzaki et al., 2004). In addition, there are several cDNA sequencing projects underway for numerous algal species. This wealth of genomic data is serving as a powerful means for the development and application of recombinant techniques for these species. Expressed sequence tag (EST) approach inferred the data about coding sequences from the whole genome. The ESTs approach has been used for the first time in red alga *Gracilaria gracilis* to identify the genes encoding for carbohydrate metabolism (Lluisma and Ragan, 1997). The progress in EST studies led to the construction of cDNA libraries for several species of red algae including *Porphyra haitanensis*, *P. yezoensis*, *Griffithsia okiensis*, *Gracilaria changii*, *G. gracilis*, *Chondrus crispus*, and *Galdieria sulphuraria* (Table 6). These cDNA libraries referred to coding genes related to metabolic pathways (Weber et al., 2004), photosynthesis, nucleic acid synthesis, repair and processing, cellular maintenance, stress responses (Lluisma and Ragan, 1997). This technique has been further utilized to understand the control of complex tri-phasic life cycle with the identification of the phase determining genes (Liu et al., 1994; Ren et al., 2006; Kakinuma et al., 2006; Collén et al., 2006, 2007). Several genomes have been completed for red macroalgae, including plastid genomes for a species of *Gracilaria* and *Porphyra* and mitochondrial genome for *Chondrus* and

Table 6. Expressed sequence tags data of red seaweeds.

Species	No. of ESTs	Reference
Porphyra haitanensis	5,318	Xiaolei et al. (2007)
Griffithsia okiensis	1,104	Lee et al. (2007)
Gracilaria changii	8,088	Teo et al. (2007)
Chondrus crispus	4,054	Collen et al. (2006)
Porphyra yezoensis	1,152	Kakinuma et al. (2006)
Galdieria sulphuraria	5,270	Weber et al. (2004)
Porphyra yezoensis	81	Lee et al. (2000)
Gracilaria gracilis	200	Lluisma and Ragan (1997)

Table 7. Genome organization of red seaweeds.

Species	Genome	Size (bp)	Reference
Galdieria sulphuraria	Nuclear	8,000,000	Barbier et al. (2005)
Gracilaria tenuistipitata	Plastid	183,883	Hagopian et al. (2004)
Cyanidioschyzon merolae	Nuclear	16,520,305	Matsuzaki et al. (2004)
Cyanidioschyzon merolae	Plastid	149,987	Matsuzaki et al. (2004)
Cyanidioschyzon merolae	Mitochondria	32,211	Matsuzaki et al. (2004)
Porphyra yezoensis	Plastid	191,952	Reith and Munholland (1995)
Chondrus crispus	Mitochondria	25,836	Burger et al. (1999)
Porphyra purpurea	Mitochondria	36,753	Burger et al. (1999)
Porphyra purpurea	Plastid	191,028	Reith and Munholland (1995)

Porphyra (Table 7). However, the efforts are underway to complete the nuclear genome of *Porphyra umbilicalis* by the US's Dept. of Energy Joint Genome Institute (JGI).

4. Conclusions

It is evident from the foregoing account that there is considerable progress made in both cellular and molecular biotechnology for red algae. The success achieved in cellular biotechnology will undoubtedly be useful in the studies of genetic transformation and bioprocess engineering of red seaweeds. The transformed cell lines can now be grown with greater confidence and success. Also, the progress made in the development of a variety of molecular markers will prove useful in elucidating the phylogenetic and evolutionary relationship among the species while aiding the algal systematics. The ongoing genomic projects will certainly provide a large pool of genetic elements such as promoters, which can be effectively used for transgene expression as well as inventory of genes that could be used as possible targets for genetic engineering programs aimed at manipulation of metabolic pathways in the near future.

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PART 4:
CYANIDIA

Castenholz
McDermott
Enami
Adachi
Shen
König
Holtgrefe
Schiebe
Reeb
Bhattacharya
Azúa-Bostus
Vicuña

Biodata of **Joseph Seckbach**, author of ***"Overview on Cyanidian Biology"*** and editor (with David J. Chapman) of this volume.

Prof. Joseph Seckbach is the Founder and Chief Editor of book series *Cellular Origins, Life in Extreme Habitats and Astrobiology* (COLE). See www.springer.com/sereis/5775. He is the author of several chapters in this series. Dr. Seckbach earned his Ph.D. from the University of Chicago, Chicago, IL (1965), and spent his postdoctoral years in the Division of Biology at Caltech (Pasadena, CA). Then, he headed a team at the University of California at Los Angeles (UCLA) for searching extraterrestrial life. He has been appointed to the faculty of the Hebrew University (Jerusalem, Israel), performed algal research, and taught biological courses until his retirement. He spent his sabbatical periods in UCLA and Harvard University, and served at Louisiana State University (LSU) (1997/1998) as the first selected occupant Chair for the Louisiana Sea Grant and Technology transfer, and as a visiting Professor in the Department of Life Sciences at LSU (Baton Rouge, LA). He spent two DAAD (German fellowships for exchange academicians) in Tübingen (1988) and recently (2006) in Ludwig Maximilians University in Munich.

Among his publications are books, scientific articles in the lines of phytoferritin, cellular evolution, acidothermophilic algae, and life in extreme environments. He also edited and translated several popular books. Dr. Seckbach is the co-author (with R. Ikan) of the *Chemistry Lexicon* (1991, 1999) and a co-editor of *Proceeding of Endocytobiology VII Conference* (Freiburg, Germany, 1998) and the *Proceedings of Algae and Extreme Environments meeting* (Trebon, Czech Republic, 2000). His new edited volume (with Richard Gordon) entitled *Divine Action And Natural Selection: Science, Faith, And Evolution* has been published by World Scientific Publishing Company. His recent interest is in the field of enigmatic microorganisms and life in extreme environments.

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OVERVIEW ON CYANIDIAN BIOLOGY

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1. Introduction

This chapter refers to the unicellular red algae, *Cyanidioschyzon, Cyanidium*, and *Galdieria* (Cyanidiaceae, Cyanidiophyceae, and Cyanidiales), as "cyanidia" (Seckbach, 1987; Lehr et al., 2007b). They are the only phototrophs and sole primary producers occurring in geothermal environment (40–56°C) and in acidic sulfur habitats (pH 0.2–4). The cyanidia are simple eukaryotic cells and asexual primitive photosynthesizers. Two species (*Galdieria sulphuraria* and *Cyanidium caldarium)* in some lab cultures thrive heterotrophically on mineral media supplied with different carbohydrates, including a number of rare sugars and sugar alcohols, as well. *Cyanidioschyzon merolae* has apparently lost ability to synthesize cell walls and to use of organic compounds (Barbier et al., 2005; Toplin et al., 2008).

Because of their simplicity and their harsh growing conditions, they should be regarded as extremophiles (Rothschild and Rocco, 2001) and as "living fossils." Their severe environments may resemble the first conditions on our planet, and therefore may be considered as pioneering eukaryotic cells on early Earth. Furthermore, *Cyanidioschyzon* is considered the smallest eukaryotic cell and perhaps the intermediate organism between the cyanobacteria and the first eukaryotic cells (Seckbach, 1987, 1994, 1995). Indeed, this alga has one of the smallest genomes (16.5 Mbp organized on its 20 chromosomes) among all photosynthetic eukaryotes (Matsuzaki et al., 2004; Misumi et al., 2005; Müller et al., 2009). The cyanidian members may also provide valuable information on the basic and essential genes involved in the lives of photosynthetic eukaryotes, including the higher plants. It is assumed that they diverged from the common tree of the Archae-plastida around one and a half billion years ago. These cells, which are distributed worldwide, thrive in isolated niches, such as in acidic sulfuric hot springs and volcanic calderas, in sulfur fumes, and within rocks (endolithic).

Modern natural history studies with *C. caldarium* started 50 years ago (Fukuda, 1958, Allen, 1959, cf. Seckbach, 1994). The chapter on *Cyanidium* by Brock (1978) provides insightful information on this alga. For more information on the cyanidia, see Seckbach (1994), Castenholz and McDermott (2009), Enami et al. (2009), König et al. (2009), and Lopez-Bautista (2009). Today, we know single species of the genus *Cyanidium* (*C. caldarium*) and of the more primitive

genus *Cyanidioschyzon* (*C. merolae*). The genus *Galdieria* has been classified into four species by morphological criteria (*G. sulphuraria*, *G. maxima*, *G. partita*, and *G. daedala*), see Seckbach (1994) and Toplin et al. (2008).

2. Origins and Evolution

It has been assumed by some scientists that life originated in a warm or hot source of water (although this is quite controversial these days, see Boussau et al., 2008). This assumption of warm/hot origins may well coordinate with the fact that the cyanidian members are simply structured primitive cells and thrive under severe conditions (pure CO_2, elevated temperature, and high level of acidity) that may resemble early environments of the young Earth (Seckbach, 1995). The postulation is that the cyanidia are among the early eukaryotic microorganisms that thrived in ancient environmental conditions and therefore may even be considered to be "living fossils."

There are a few models for eukaryogenesis and evolution of the cyanidia as the pioneers in the primary Eukaryota. The main hypotheses are endocytosymbiosis and direct filiations (or compartmentalization). The more widely accepted concept is that of endosymbiosis. Castenholz (1979) proposed an interesting association between a colorless *Chlorella* and a cyanobacterium as the initial "components" of *C. caldarium*. In this volume, Castenholz and McDermott (2009) have suggested that an originally achlorotic (plastids absent) eukaryotic host incorporated a thermophilic cyanobacterium as its endosymbiont. The association of thermophilic cyanobacteria and their hosts, which maintain an internal pH close to neutrality, creates a comfortable climate for all the cyanobacteria, which turn in time into chloroplasts.

Seckbach and Walsh (1999) and earlier Seckbach et al. (1993a) proposed that the *Cyanidioschyzon* cell (lacking a cell wall) might be the result of the association between a host thermophilic cell (*Thermoplasma*-like archaean microbe) and a small cyanobacterium. The *Cyanidioschyzon* perhaps proceeded to evolve via compartmentalization process into *C. caldarium* (which developed its heavy cell wall), and the latter cell, led into the various species of *Galdieria* (Seckbach et al., 1983; Seckbach, 1987, 1999). Such gradual intrafamily evolution could also be supported by the cellular morphology, ultrastructural characteristics, and storage glucans of these genera (Seckbach, 1987, 1994, 1995; Seckbach and Walsh, 1999, see there table 3).

The storage glucans produced by the cyanidian enzymatic systems and stored in the cytosole (as in the red algae and chromophytes) also show a gradual development (from cyanobacterial type towards rhodophytan glucans). Highly branched phytoglycogen, similar to that synthesized by cyanobacteria, occurs in *Cyanidioschyzon.* These cells divide asexually into internal daughter cells (Table 1). This oval cell divides via binary cytokinesis. The glycogen storage material is also accumulated in *Cyanidium*, which is more advanced, and multiplies by cell division into four daughter cells endospores). *Galdieria* synthesizes only floridean starch (close to the amylopectin of

Table 1. Main characteristics of the three cyanidian species.*

Feature	*Cyanidioschyzon merolae*	*Cyanidium caldarium*	*Galdieria sulphuraria*
Shape	Oval, club-like	Spherical	Spherical
Size (μm)	1.5–3.5	2–5	3–16
Division via	Binary fission	Four endospores	4–32 endospores
Heavy cell wall	Absent/very fragile	Present	Present
Chloroplast	One-polymorphic	One-spherical	One-multilobed
Plastid DNA shape	Bead-like	Rod-shaped	Ring-shaped
Plastid DNA region	Central	Central	Peripheral
Plastid DNA amount ($\times 10^4$ phons)	8.3	72.8	231
Mitochondria	One-inconspicuous	Concave lens	Multilobed/net-like
Microbodies	Usually invisible	Present	Few in full view
Storage glucans	Phytoglycogen	Phytoglycogen	Floridean starch
Linolenic acid	Absent	Absent	Present
N-form uptake as	$NH_4 + NO_3$	$NH_4 + NO_3$	Only NH_4
Salt tolerance (%)	3	3–4	10
pH optimus	1.5	1.5	2.0
Nutritional patterns	Autotrophic	Autotrophic	Auto and heterotrophic
Nuclear DNA content			
($\times 10^6$ Bp)	8	13	25–48,7
($\times 10^4$ photons)	104	193	378
Vacuoles	Absent	Absent	Present
Number of species	1	1	4

*Based on Seckbach and Walsh (1999).

Rhodophyta) and this alga divides into 4, 8, or up to 32 daughter cells within the mother cell walls (Table 1, Fredrick, 1993; Seckbach et al., 1993a; Seckbach and Walsh, 1999). It has been published that this alga tolerates desiccation as it has been observed in dry endolithic layers (Müller et al., 2009). On the other hand, Castenholz found no desiccation tolerance in over 120 isolates of cyanidia (pers. comm.). Kuroiwa et al. (1994) (cf. Seckbach et al., 1993a) also portrayed an evolutionary tree of various algae, on the basis of their chloroplast nucleoids' organization and "planted" the cyanidian algae in the same ascending sequence (Cyanidioschyzon → Cyanidium → Galdieria → Rhodophyta → Chromophyta).

3. Habitats

Cyanidian cells tolerate and withstand the extreme stress of heat (up to 56°C), acid (pH 0–4), and salts (up to 10% solutions), and proliferate even when exposed to a stream of 100% CO_2 (Seckbach et al., 1970; Kurano et al., 1995), and tolerate heavy metals (Castenholz and McDermott, 2009). It has been reported that these cells resisted being submerged in 1 N H_2SO_4 (Allen, 1959), which is a practical method for purifying cultures in the lab and getting rid of other microbial contaminations. It was determined that the inner pH of the acidophilic cells within the cytosol is

kept at close to neutral, as also occurs in other acidophilic algae (Seckbach and Oren, 2007). The microalga *G. sulphuraria* (of this group) can represent up to 90% of the biomass in extreme habitats, such as hot sulfur springs with pH values of 0–4. This alga tolerates desiccation (Müller et al., 2009) and high concentrations of toxic metal ions such as cadmium, mercury, aluminum, and nickel, suggesting potential application in bioremediation (Weber et al., 2007). Likewise, Lehr et al., (2007a) have demonstrated the oxidation of antimony and arsenic compounds by members of cyanidia.

In addition to the cyanidian members that occur in acidic habitats of hot aqueous water and in dry volcanic places, cells were also observed in crypto-endolithic layers of rocks (Gross et al., 1998; Yoon et al., 2006a; Pinto, 2007; Pinto et al., 2007; Toplin et al., 2008). They form a green band, sandwiched between the surfaces of the rocks, and absorb lower intensity of illuminations to photosynthesize (0.1–1% of the sunlight reach the upper part of the surface layer). Gross et al. (1998) proposed that endolithic cells (of *Galdieria*) grow in low light intensities, might be also grown as heterotrophs, and nourish themselves from compounds released from the dead cells in the subsurface layer. These endolithic cyanidia have been observed in various places (Gross et al., 1998; Ciniglia et al., 2004; Walker et al., 2005; Yoon et al., 2006a, Pinto, 2007; Pinto et al., 2007; Toplin et al., 2008; Castenholz and McDermott, 2009; Reed and Bhattacharya, 2009).

4. Cave *Cyanidium*

Azúa-Bustos (2009) described nonthermophilic species of *Cyanidium*, which grow in the Atacama Chilean caves. Such species were described earlier by Hoffmann (1994, see his references to Schwabe) and a few others. Similar cave *Cyanidium* cells were observed in caves in Jerusalem and southern Italy as well as in a limestone cave in France (see references of Hoffmann, 1994). The cave *Cyanidium* cells are mesophilic members of the *Cyanidium* clade. Such nonthermophilic and also nonacidophilic *Cyanidium* cells evolved probably into their acidothermophilic habitats by escaping competition in their ambient environment by moving into their extreme habitats (Toplin et al., 2008). They grow under low intensity of about 0.06–0.17% of the external incident light. Azúa-Bustos (2009) reports that these cells are in acidic environments (pH 4.5), similar to their "relatives" in the cyanidian family; however, they thrive at around 14°C, which removes their thermo features from the thermophilic "cousins." He also compared some genes of these cells (from other caves) with known cyanidian members (Azúa-Bustos, 2009). These cells have a phylogenetic origin of quite ancient record of ~1.3 Bya. Their microscopic morphology and ultrastructure are close to *C. caldarium,* with their chloroplast concentric thylakoid membranes containing numerous embedded phycobillisomes. For more details, see Azúa-Bustos (2009). Gross et al. (2002) also showed that *Galdieria* has been isolated from nonthermophilic (optimum growth at 30°C) soil samples in Soos (Czech Republic).

5. Distribution

Toplin et al. (2008) suggested that the cyanidia may have been distributed by the birds' digestive system (as has been determined for the dispersal of green algae, diatoms, and cyanobacteria) or by mud on their feet. *C. caldarium* and its cohorts (Cyanidioschyzon and Galdieria) are the only photosynthetic organisms found in hot-acidic environments and represent the sole primary producers in these ecosystems. They are universally distributed in acid hot media (in aqueous and terrestrial locations). These algae have been observed in Yellowstone (USA), southern Italy, Japan, New Zealand, Iceland, and elsewhere. Moreover, other Cyanidium cells have been found in caves in Chile and other countries (see above and in Seckbach 1994). Studies made in caves of the Atacama Desert in Chile (Azúa-Bustos, 2009) provide more information on these Cyanidium. The cyanidian members have been observed in caves where only 0.06% of the external incident light penetrates. Similar light effect occurs in Pozzuoli (Naples, IT) where the *C. caldarium* grows under the lime layer adjacent to volcanic vapor, gases, and fumaroles. Another chapter on cave *C. caldarium* appears elsewhere (Seckbach, 1994).

6. Taxonomy and Phylogeny

The cyanidia are an asexual taxon in the Rhodophyta (red algae) in spite of their not being red but rather green to blue-greenish owing to their predominant photopigments, i.e., C-phycocyanin, allophycocyanin, and chlorophyll-a. Historically, published data on this microbial group have been confusing and even inaccurate (Seckbach, 1994; Pinto, 2007). This misinformation has led to a puzzling picture of cyanidian taxonomy, phylogeny, ecology, physiology, morphology, and genetics (Seckbach, 1992, 1994). The reason for this confusion originated from the fact that all studies dealt with the whole population of Cyanidiaceae. So, the algal cultures contained the three genera in one exanimation, and mistakenly these researchers considered it as uniculture of sole *C. caldarium*. The taxonomic position of *C. caldarium* was unresolved for a long period; it has been considered variously as a cyanobacterium and as anomalously pigmented chlorophyte (Allen, 1959; Seckbach, 1972; Seckbach and Ikan, 1972; Chapman, 1974; Ueda and Yokochi, 1981), others considered it as an algal that occupies a transitional position between the cyanobacteria and the Rhodophyta (Holton, 1973; Fredrick, 1976; Seckbach and Fredrick, 1980; see Yoon et al., 2006b). There were suggestions that *C. caldarium* is a colorless eukaryotic alga containing an endosymbiotic cyanelle (Kremer et al., 1978; Kremer, 1982, and cf. Ford, 1984). This confusion in algal taxonomy ended with the publications of the Neapolitan cyanidian researchers, in which they finally clarified the problem at least temporarily. They have "solved" the "*C. caldarium*" puzzle and divided the group into three genera, namely *C. merolae, C. caldarium,* and *G. sulphuraria* (Deluca and Taddei, 1976; Merola et al., 1981; see Pinto et al., 1994). However, more has to be done in the taxonomic issue with future genetic studies. The main

characteristics of the three cyanidian genera (Table 1) have been published elsewhere (Seckbach, 1987, 1992; Seckbach et al., 1983; Seckbach and Walsh, 1999).

Owing to the primitive features and the early evolution of the cyanidian cells, Seckbach suggested placing them in a new taxon of PreRhodophyta (Seckbach, 1987, 1992, 1994, 1999; Seckbach and Ott, 1994). We have to admit that this term has not been adopted – rather Cyanidiophycease.

For further discussion on the taxonomy and phylogeny status of cyanidia, see Seckbach (1991, 1994), Ott and Seckbach (1994), Albertano et al. (2000), Ciniglia et al. (2004), Müller et al. (2009), and Yoon et al. (2006b).

7. Morphology and Ultrastructure

In the past, "*C. caldarium*" showed various appearances under the microscope, and those phases of the "*Cyanidium"* have been called by various names. Then, it turned out that the "*Cyanidium*" culture also contained *Galdieria* (and *Cyanidioschyzon)*, which were the reason for the multiple faces of "*Cyanidium*." Several ultrastructural studies have been performed in the past on the cyanidiaceaen cells (Seckbach et al., 1981, 1993a; Ford, 1984; Seckbach, 1987, 1992; Pinto et al., 1994; and chapters by Ueda and by Kuroiwa et al., in Seckbach, 1994; Pinto, 2007). These algal cells contain a nucleus, chloroplasts, and mitochondria; in *Cyanidium* and *Galdieria*, there are also microbodies and vacuoles. Chloroplasts and mitochondria of the cyanidia divide and multiply with a dividing ring process (Kuroiwa et al., 1994; Miyagishima and Nakanishi, 2009), so that Cyanidiaceae division may serve as a target for studies of the structural dividing mechanism. The chloroplasts contain parallel rows of unstacked thylakoids surrounded by a peripheral thylakoid; concentric thylakoids have also been observed (see above references).

8. Biochemistry and Physiology

It has been assumed that more than 50% of the total photosynthesis occurs in aquatic microorganisms in marine and freshwater environments. The cyanidiaceaen cells thrive in laboratory cultures under bubbling of pure CO_2 (Seckbach et al., 1970) and show higher yield (photosynthesis rates and number of cells) than those grown under streams of air. The photosynthetic pigments of this group are chlorophyll-a, allophycocyanin, and C-phycocyanin (they do not possess the red pigment phycoerythrin nor any chlorophyll-b), hence their blue-green coloration. The cyanidiaceaen enzymes may serve in algal biotechnology and pharmaceutical applications. They provide thermostable enzymes and small molecules (such as heteroside floridosides). In addition, they provide thermotolerant proteins and share with the other red algae production of important commercial and biotechnological products.

The cyanidia produce storage carbohydrates, namely, glycogen and floridian starch (Fredrick and Seckbach, 1986; Fredrick, 1993). Seckbach et al. (1992)

reported on the presence of peroxisomes in the cyanidia (see Raven, 2009) as well as sterols (Ikan and Seckbach, 1972; Seckbach et al., 1993b). See also Gentry et al. (2009) who discussed the laforin from *C. merolae*, which has properties similar to human laforin (related to epilepsy and death).

9. Molecular Biology – Genomic Study

Recent genomic studies have shown interesting results (Toplin et al., 2008; Stanke, 2009; Lopez-Bautista, 2009). Over the past 40 years, the cyanidian studies have shifted from an unknown and enigmatic algal group into the genomic modern popular age (Ciniglia et al., 2004). Currently, owing to the genomic investigations, these cells have gained much interest and popularity in the literature. Members of these microalgae have been sequenced of late, and during the past few years, research on red algae has moved into the genomics age. The genomes of two key members of the cyanidia (*Cyanidioschyzon* [complete genome] and *Galdieria* [almost complete]) have been sequenced (Barbier et al., 2005; Misumi et al., 2005; Weber et al., 2007; Castenholz and McDermott, 2009; Gantt et al., 2009). These breakthrough events have opened new avenues for comparative genomics, large-scale functional genomics studies, and molecular evolutionary and phylogenomics studies. The investigations of the *Cyanidiaceae* are on their way to making them important model organisms in Systems Biology and possible models for extraterrestrial life. In summary, we may conclude that *C. Caldarium*, which has been considered an ugly duckling, has turned out to be a beautiful algal swan.

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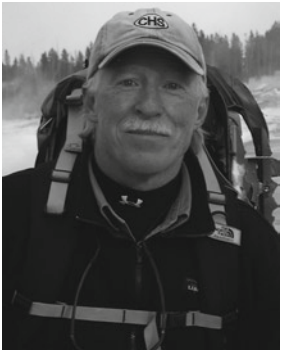

THE CYANIDIALES: ECOLOGY, BIODIVERSITY, AND BIOGEOGRAPHY

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1. Introduction

The order Cyanidiales (or class Cyanidiophyceae) is comprised of asexual, unicellular red algae that are known to grow in low pH environments (0.2–3.5 or 4.0) and at moderately high temperatures (up to 56°C) and are typically found in acidic geothermal habitats throughout the Earth. No other photosynthetic microorganisms are known to inhabit this combination of conditions. The order Cyanidiales, since 1981, is thought to consist of three genera: *Cyanidium*, *Galdieria*, and *Cyanidioschyzon* (Ciniglia et al., 2004; Gross et al., 2001; Heilmann and Gross, 2001). This group of algae appears to be phylogenetically quite distinct from the main line of descent in the red algae and branches off quite early in geologic time (i.e. ~ 1.3–1.4 Ga), based on phylogenetic, molecular clock inference and fossil evidence for the first reputed macroalgae, which are presumed to be ancestors of the Rhodophyta (Yoon et al., 2002, 2004, 2006b). In this chapter, we comment on the ecology, biodiversity, and biogeography of these fascinating eukaryotic extremophiles, attempting to assimilate recent, important developments in our understanding of these algae.

2. Aspects of Cyanidiales Ecology

Prime habitats include warm-hot acidic springs and pools that emanate from geothermal sources, solid substrates bathed by steam from such pools, rock surfaces surrounding solfataras, and soils, gravel, and in crusts or as endoliths in such areas. Cyanidiales may even occur surrounding alkaline springs, bathed in steam where H_2S is oxidized to sulfuric acid (R.W. Castenholz, 2002–2004, unpublished data). A few taxa of the Cyanidiales also occur in nonthermal acidic habitats (Gross et al., 2002), although at least one of these strains has retained its thermophilic properties (Gross and Gross, 2001). No other phototrophs are expected to be in this combination of low pH and elevated temperature, and thus such environments demarcate

the environmental niche for the Cyanidiales. It is well known that cyanobacteria and other phototrophic prokaryotes do not occur in volcanic waters (warm or cold) below about pH 4, and only few species occur below pH 5 (Ward and Castenholz, 2000).

Results of recent, ecology-oriented studies have begun to shed light on what might be specific habitat preferences for the different genera. Environmental surveys, using PCR cloned *rbcL* sequences, have found that populations represented by *Galdieria*-A, *Galdieria*-B, and *Cyanidium* phylotypes appear to prefer, or at least are capable of withstanding, the rigors of an endolithic environment (Ciniglia et al., 2004; Yoon et al., 2006a). Interestingly, *Cyanidium* has not always been known to co-inhabit the endolithic and interlithic environments with *Galdieria* (Ciniglia et al., 2004; Yoon et al., 2006a). In these studies, *Cyanidioschyzon merolae* populations were encountered only in very humid or moist environments. Further detailed studies are necessary to determine if this was simply a sampling coincidence or if there are site-specific features that are important to various types of the Cyanidiales, although in extensive cultivation work in Yellowstone (>140 isolates from 20 disparate locations), we have observed similar trends and although *C. merolae* morphotypes were isolated from a soil crust at one site (Toplin et al., 2008). Usually, only *Galdieria*-*Cyanidium* morphotypes were isolated from these habitats. Surprisingly, thus far we have not yet isolated a bona fide genetically determined *Cyanidium* from any Yellowstone geothermal feature (Toplin et al., 2008). Our efforts in this regard continue.

The Cyanidiales are blue-green to green or even yellow-green in color due to the relative proportions of the predominant chloroplast pigments, c-phycocyanin, allophycocyanin, chlorophyll *a*, and a few carotenoids, all of which also occur in the cyanobacteria. However, phycoerythrin has not been found in these algae (Lin et al., 1990). The robust pigment composition of these algae and their very significant mat presence establish them visually as a dominant component of the microbial communities that inhabit the less extreme regions of thermal gradients in acidic geothermal springs (e.g. Fig. 1). Water temperatures in submerged mats can range between 38°C and 56°C, although in these particular environments the most robust mats typically are associated with water temperatures around 42°C (unpublished data) or 45°C (according to Brock, 1978).

The non-photosynthetic, acidophilic bacteria and archaea associated with these algae are poorly understood. Jackson et al. (2001) used PCR coupled to denaturing gradient gel electrophoresis to provide a qualitative assessment of the bacterial and archaeal diversity occurring in the outflow channel of a Yellowstone acidic geothermal spring referred to as Dragon Spring. Not unexpectedly, apparent diversity increased as temperature decreased, with some of the greatest diversity occurring in the green mat region dominated by the Cyanidiales. A more descriptive PCR-based study by Walker et al. (2005) followed, identifying many bacteria and archaea in Cyanidiales-inhabited endolithic samples taken immediately adjacent to Dragon Spring. *Mycobacterium*, an abundance of Proteobacteria groups, and *Sulfolobus* and *Leptospirillum* were detected in 16S rRNA gene PCR clone

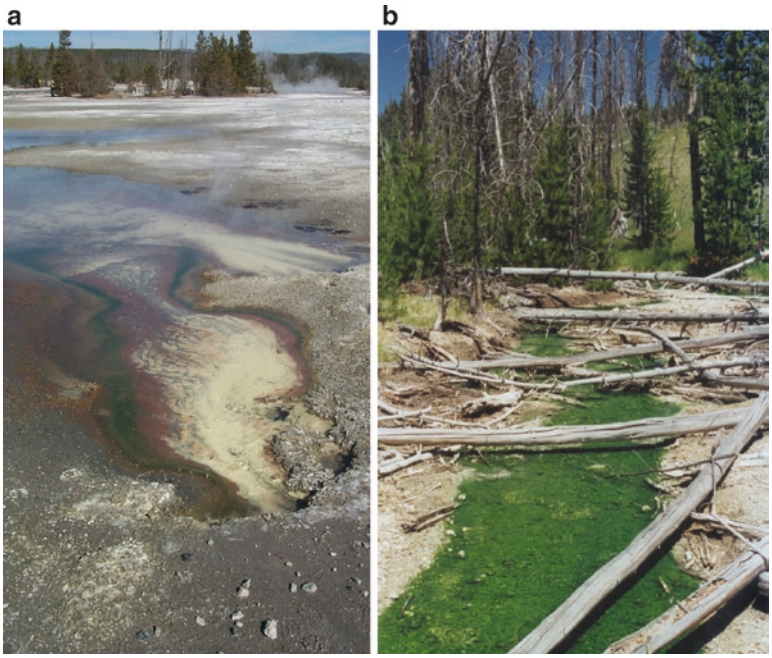

Figure 1. Photographs of Cyanidiales mats occurring in Yellowstone National Park. (**a**) Narrow green bands along the perimeter of Dragon Spring in Norris Geyser Basin. (**b**) Lemonade Creek located in the Amphitheater Springs area.

libraries. Additional studies have documented other eukaryotic microbes occurring with the Cyanidiales, but only in cooler regions of the geothermal outflows (ca. <39°C). Here the Cyanidiales habitat begins to overlap with that of other algae, such as *Chlorella* and *Paradoxia*-like algae (Ferris et al., 2005), as well as species of *Chlamydomonas, Dunaliella, Viridiella, Euglena,* diatoms (Gross, 2000), and the filamentous green alga, *Zygogonium.* It is also well known that some species of fungi occur abundantly in cyanidial mats (Brock, 1978).

Heavy metals are common in the Cyanidiales habitats, although their concentrations can vary considerably. Waters in geothermal aquifers leach various minerals from aquifer wall rocks, resulting in high levels of silica, carbonates, and metals being brought to the surface. The acidic environment of Cyanidiales helps maintain the solubility of these metals at lower surface temperatures, although metal toxicity may actually be less at lower pHs, with the opposite true for toxic ions such as arsenite and selenite (Gross, 2000). *Cyanidium* has shown a high tolerance to Al (up to 200 mM) (Yoshimura et al., 1999, 2000), and *G. sulphuraria* can tolerate Hg concentrations up to ~10 mM; roughly tenfold greater than *Cyanidium* (Pinto and Taddei, 1986). Other differences in heavy metal tolerance have been noted between *Galdieria* and *Cyanidium*. Albertano and Pinto (1986) reported that *Cyanidium* and *C. merolae* are more tolerant of arsenic, mercury,

and copper than *Galdieria*. Mercury has been documented to be present at extraordinarily high levels in Norris Geyser Basin in Yellowstone (Phelps, 1980), and indeed, we have documented total Hg levels in a Norris geothermal soil to be >200 μg/g-soil, and from which we have isolated *Galdieria sulphuraria* and another *Galdieria*-like type (Toplin et al., 2008). Recently, we have begun to examine the interactions of these algae with the toxic metalloids, arsenic and antimony. An isolate exhibiting 99% identity (both *rbcL* and 18S rDNA) to *Cyanidioschyzon merolae* (but with *Galdieria*-like morphology) obtained from Dragon Spring, is capable of oxidizing arsenite and antimonite (Lehr et al., 2007b). Further, ongoing studies concerning arsenic methylation (unpublished data) suggest that the Cyanidiales may be important contributors to the very significant arsenic methylation occurring in Yellowstone (Planer-Friedrich et al., 2006), and that process has been observed primarily in acidic habitats (Planer-Friedrich and Merkel, 2006). Thus, it would seem that these algae may contribute more to microbial community function in the geothermal setting than simply primary production.

UV radiation is well established as inhibitory to algae (e.g., Holm-Hanson et al., 1993), and two Yellowstone studies have examined the influence of UV irradiation on the Cyanidiales. Cockell and Rothschild (1999) did not find evidence of UV-based photosynthesis inhibition in the Cyanidiales that colonize Nymph Creek, a heavily shaded geothermal environment located in the Norris-Mammoth corridor. The use of mat cores in their study may have masked the actual effects of UV on cells exposed on the surface of the mat. In contrast, monthly measurements during a year-long study at Dragon Spring in the Norris Geyser Basin obtained several different lines of evidence that were all consistent with the suggestion that UV irradiance is likely a keystone environmental factor for these algae (Lehr et al., 2007a). Dragon Spring is located in an open, completely unshaded area, and thus is exposed to long photoperiods during the summer months, which coincides with maximum solar intensities and a major mat disturbance event referred to as "mat decline" (Lehr et al., 2007a) (Fig. 2). Various chemical and physical measurements showed that viable counts of Cyanidiales (assessed using the most probable number technique (MPN)) were significantly positively correlated with temperature and negatively correlated with UV/Visible irradiance (Lehr et al., 2007a). Additional measurements in that study also showed that photosynthesis is significantly inhibited by UV-A and UV-B exposure, and thus demonstrated evidence that the mat decline phenomenon is more closely related to UV exposure as opposed to visible light, although high intensities of visible radiation can also be inhibitory to members of the Cyanidiales (Brock, 1978). Furthermore, a comparison of PCR-generated 18S rRNA clone sequences and microsatellite sequences derived from DNA extracted during the mat decline period (July) versus those obtained when MPN counts were at a maximum (October) showed clear evidence that these major mat changes were accompanied by shifts in types dominating the population (Lehr et al., 2007a). Potentially, UV adaptation or sensitivity could contribute to diversification and/or to habitat preference (e.g., endolithic or soil habitats).

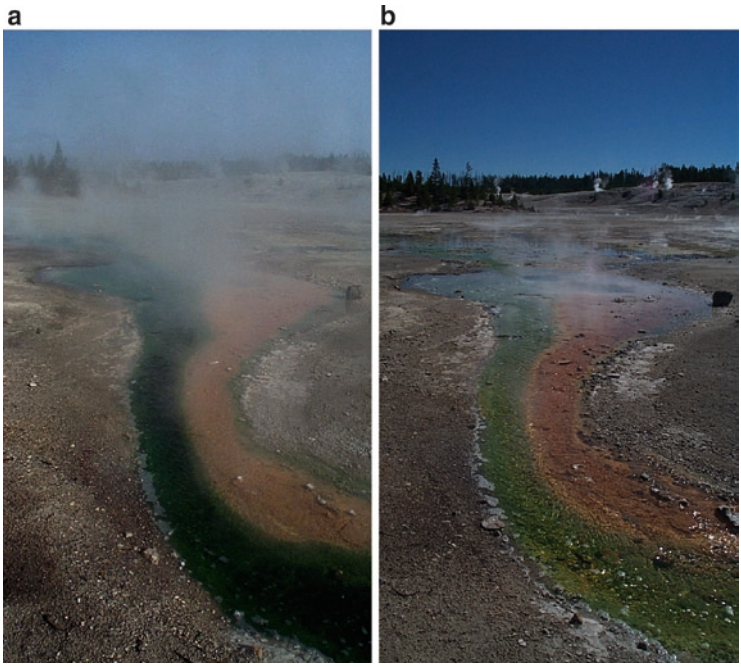

Figure 2. Photographs documenting mat decline during the long photoperiods of summer. Geothermal spring flow merging with that derived from Pinwheel Geyser (during a quiescent period) located along the boardwalk at Norris Geyser Basin. (**a**) January, 2005 and (**b**) early July, 2005. (Reprinted with permission from the *Journal of Phycology.*).

3. Biodiversity

Confirmation of the relatedness of the cyanidia to the red algae was produced without the use of genetics with the identification of floridosides in all three genera in addition to other red algal components (De Luca and Moretti, 1983). However, the cell wall (when present) is largely (50–55%) proteinaceous, unlike most of the red algae (Bailey and Staehelin, 1968). With only a single membrane surrounding the plastid, it was determined to have been the result of a primary endosymbiosis (De Luca and Moretti, 1983). There is no doubt now that the Cyanidiales form a distinct phylogenetic clade. However, for many years *"Cyanidium"* was classified as a cyanophyte or a green alga with anomalous pigmentation, and so it was when M.B. Allen isolated the first culture (Allen, 1959). A more complete history of these sequential events may be found in Brock (1978), Pinto et al. (1994), and Pinto (2007). Important recent developments in the study of these algae include the sequencing of the complete genome of *Cyanidioschyzon merolae* (Matsuzaki et al., 2004) and a nearly complete genome of *Galdieria sulphuraria* (Barbier et al., 2005).

The complete genomes of the chloroplast (Ohta et al., 2003) and mitochondrion (Ohta et al., 1998) of *C. merolae* have also been published.

The type genus and species *Cyanidium caldarium* was first described in 1933 as a cyanophyte (cyanobacterium) by Lothar Geitler (Geitler, 1933) and unknowingly later by Joseph Copeland in 1936, again as an unusual cyanophyte and named *Pluto caldarius* (Copeland, 1936). An organism referred to as *Cyanidium caldarium* was studied extensively by Doemel and Brock (1971, 1978), but at that time there had been no division into three genera and all members of this group were referred to as *Cyanidium caldarium.* The Doemel and Brock studies were probably using *Galdieria sulphuraria*, if indeed only one "type" was involved. Since then and until molecular methods had been applied, the three recognized genera and several species have been distinguished mainly by morphology and reproductive patterns. Taxa of the genus *Cyanidioschyzon* are smaller (usually 1–2 μm in breadth) with an oval, club- or pear-like shape (see Matsuzaki et al., 2004). They reproduce by binary cytokinesis and lack a rigid cell wall. *Galdieria* and *Cyanidium* have a more spherical shape, a rigid cell wall, and reproduce through the formation of 4–32 small daughter cells within a mother cell. Some types have also been distinguished by physiological characteristics. For example, isolates of *G. sulphuraria* have the ability to grow heterotrophically in the dark on at least 50 different carbon sources, including several sugars, sugar alcohols, amino acids, and TCA cycle intermediates (Barbier et al., 2005; Gross and Schnarrenberger, 1995; Oesterhelt and Gross, 2002). This characteristic of *G. sulphuraria* has been viewed as a probable explanation for its ability to grow in darkness or semi-darkness in soil, gravel, and as endolithic populations, using organic carbon released from other microorganisms in these habitats (Ferris et al., 2005; Gross and Schnarrenberger, 1995; Oesterhelt and Gross, 2002). *G. maxima* may also grow heterotrophically, although poorly according to Gross et al. (2001). *G. sulphuraria* lacks the ability to use nitrate as the sole nitrogen source, but a few other *Galdieria*-like isolates also lack this ability (Toplin et al., 2008).

Thermo-acidic environments are scattered disparately like small islands throughout the Earth. Since members of the Cyanidiales tested thus far (>30) do not tolerate desiccation (Toplin et al., 2008), the geographical separation and isolation of thermo-acidic sites may have led to allopatric speciation events over time (Gross et al., 2001). One large geothermal "island" with many acidic habitats is the Yellowstone geothermal region, an approximately 9,000 km^2 area with numerous hydrothermal features that vary in pH, temperature, exposure to high solar radiation, water availability, and concentrations of soluble metals and metalloids (Nordstrom et al., 2005). Any of these physico-chemical factors could exert selection pressures, potentially providing opportunities for distinct species or ecotypes to arise even within an area as small as YNP. Indeed, evidence of habitat specialization has been found for the thermophilic cyanobacterium *Mastigocladus (Fischerella) laminosus* within YNP (Miller et al., 2006), *Synechococcus* (Ward et al., 2006, Bhaya et al., 2007), and *Sulfolobus* (Whitaker et al., 2003).

Cultivation-independent work conducted in Italy has identified novel *rbcL* phylotypes representing a novel lineage referred to as *Galdieria*-B (Ciniglia et al., 2004). Our studies have used both genetic and morphological criteria to examine

the diversity of a large number of isolates of the Cyanidiales obtained from 13 distant and chemically disparate sites within Yellowstone, and from larger spatial scales that include Japan, New Zealand, and Iceland. Our most common Yellowstone isolate appears to be a *Galdieria* or *Cyanidium* morphotype, since it possesses a cell wall and divides internally to produce an even number of small daughter cells that eventually are liberated from the mother cell. Based on the 18S rDNA and *rbcL* markers, however, these cells show 99–100% identity to the morphologically very different *Cyanidioschyzon merolae* (Toplin et al., 2008). Although morphology alone can differentiate the genus *Cyanidioschyzon* from *Galdieria* and *Cyanidium* (see above), at this point only genetic means can distinguish taxa of this order that, in some cases, may be so distantly related that they may constitute new, previously undescribed species. The situation with the aforementioned Cyanidiales isolates is similar in many ways to the problem with unicellular microorganisms in general. Comparisons of the sequences of one or two genes may be inadequate. Other genes or their products or partial or complete genomes are needed to further establish the genetic relatedness of this novel member of the Cyanidiales. Microorganisms that are identical, based on 18S rRNA gene sequence criteria, but which are distinctly different species is not without precedence (e.g., in dinoflagellates, Logares et al., 2007). Short sequence repeats (i.e., microsatellites) have been useful for identifying genetic diversity among these isolates (Lehr et al., 2007a; Toplin et al., 2008), but at this point there is no meaningful correlation between these variants and specific habitat characteristics (Toplin et al., 2008). It is important to note that indisputable *C. merolae* morpho-phylotypes also occur in Yellowstone and have also been cultured (Toplin et al., 2008).

4. Biogeographical Considerations

Apart from the quandary of how to characterize and name this provisional new species or variety that is incorrectly identified as *Cyanidioschyzon merolae* (using only the sequences of two genes), there are also major subgroups of the Cyanidiales in other distant provinces (i.e., Japan, New Zealand, and Iceland) that do show genetic distinctiveness, particularly using chloroplast *rbcL* gene sequences, and none of which show closest identity to *C. merolae.* For the isolates from these spatially disparate locations, at this time the use of only 18S rDNA and *rbcL* sequences seems adequate to point out several taxa quite distinct from the two major groups in Yellowstone (see Table 1). As indicated by phylogenetic analysis (one based on *rbcL,* the other on 18S rDNA sequences), there are three distinct groups (clades) of isolates from Japan that show closest identity to an isolate from the Kurile Islands (off the northeast coast of Hokkaido, Japan) named *Galdieria maxima* (Table 1) (Toplin et al., 2008). Similarly, three phylogenetic clades from New Zealand also show genetic separation from the Yellowstone isolates and from the Japanese strains (the latter at least at the *rbcL* locus) (Toplin et al., 2008). In contrast, eight isolates from several sites in southwest Iceland were identical to each other at both the *rbcL* and 18S rDNA loci, and like one

Table 1. Nearest BLAST relative for each type of isolate from YNP, Japan, New Zealand, and Iceland for rbcL. The number in the parentheses is the % identity to the nearest GenBank relative. The percent variation for each group is also listed for each gene, followed by the number of isolates compared (n). (Modified from Toplin et al., 2008.)

	Nearest Blast Relative rbcL	% Variation Within Group + number of strains analyzed
YNP		
Type IA/IB	*C. merolae* 10D (99)	0.38, n =16
Type II	*G. sulphuraria* UTEX 2393 (99)	0.38, n =5
Japan		
Type IIIA	*G. maxima* IPPAS507 (99)	1, n = 9
Type IIIB	*G. maxima* IPPAS507 (96)	1, n = 14
Type IIIC	*G. maxima* IPPAS507 (93)	0, n = 1
New Zealand		
Type IV	*G. sulphuraria* SAG 108.79 (96)	0.8, n = 10
Type V	*G. maxima* IPPAS P507 (93)	1, n = 8
Type VI	*G. maxima* IPPAS P507 (91)	0, n = 1
Iceland		
Type VII	*G. maxima* IPPAS507 (96)	0, n = 8

particular clade from Japan, showed the closest identity to *Galdieria maxima* (E. Perry et al., 2008, unpublished data).

The most cosmopolitan of all cyanidial "species", *G. sulphuraria* has also been isolated (3 strains) from endolithic habitats in southwestern Iceland (Gross and Oesterhelt, 1999). How did cyanidia find their way to Japan, New Zealand, and Iceland from the Yellowstone or Italian (or other) geothermal areas or vice versa? Indeed, it may be difficult to identify the ancestral source of the Cyanidiales evolutionary clade. If the Cyanidiales with its present general characteristics is as ancient as indicated by molecular clock inference, dissemination difficulties may not be so relevant, since the disparate geothermal "islands" may represent remnants of an origin that occurred long before present continental positions existed. Still, however, issues of dispersal still exist, since many volcanically active island sites that support populations of Cyanidiales came into existence in more recent geologic times (e.g., Azores, Iceland). However, members of the Cyanidiales appear to be absent in their appropriate temperature zone in volcanic areas of Hawaii (Brock, 1978). Identifying the origin of the Cyanidiales may prove difficult. However, the most likely method of dissemination may be better understood through experimentation. We (and other authors) have been unable to show desiccation tolerance (at less than ~90% relative humidity) that would permit aerial transport of these algae over great distances (>2,000 km) with no suitable habitat intervening. However, testing pure cultures may not be a suitable method. Certainly, to successfully colonize a newly available volcanic site, enough cells to constitute a viable founder population would be necessary. It is possible that small amounts of moist acidic mud containing viable

cells could be transported by birds, or even more likely, cells could be carried in the alimentary track of water birds. It was shown many years ago that this is possible even in the case of algae that do not produce resting cells or spores (Proctor, 1959).

Nevertheless, little is known of the migratory or accidental movement of water birds in the distant past. Since Yellowstone, Japan, and New Zealand have had continuous volcanic, geothermal activity for several millions of years, a very rare transport of cells of the Cyanidiales could, nevertheless, have occurred with subsequent speciation events, particularly if somewhat different chemical environments might have selected for new varieties. Preliminary evidence indicates that a great overlap of similar acidic habitats occurs in all of these major volcanic locations. If dispersal of this type of organism is very easy and frequent for reasons we do not understand, we might expect a greater similarity among isolates from distant sites. Since it is agreed that light-exposed sites in Iceland have been exposed for colonization for less than 10,000 years after complete glaciation, it is not unusual that there is little apparent variation among the Cyanidiales of Iceland, suggesting that the time for proliferating founder populations to evolve new species was insufficient (Gross and Oesterhelt, 1999; E. Perry et al., 2008, unpublished data). There is a considerable danger currently that thermobiologists could be the most likely transport vectors. Thus, it is very important that these biologists sterilize their equipment and boots before moving from one major thermal geographic area to another. In our case, a new pair of boots has been purchased in all cases.

5. Evolution of the Cyanidiales

The Order Cyanidiales or the class Cyanidiophyceae (depending on the hierarchy chosen by different authors) constitutes a very early and distinct branch of the Rhodopyta (red algae), presumably with chloroplasts similar to the primordial chloroplast for all photosynthetic eukaryotes (Yoon et al., 2002, 2006b). Evidence indicates that it was a primary endosymbiosis. A somewhat novel hypothesis, however, is that the eukaryotic "host" of the cyanidial lineage may indeed have been quite ancient (acidophilic or not), and that this group was originally achlorotic (plastids absent), and that a thermophilic cyanobacterial endosymbiont was incorporated, and that by its presence (and genes) imparted thermophily to ancestors of the Cyanidiales. Of course, what we mean by later, is an endosymbiosis possibly during the late Proterozoic ($>1 \times 10^9$ years ago). From what we know of present day thermophilic cyanobacteria, they do not tolerate acidic conditions (Kallas and Castenholz, 1982a, b). Thus, the harboring of a thermophilic cyanobacterial symbiont in a host that maintains a pH close to neutrality creates a pH climate that is acceptable for essentially all cyanobacteria. Since it is agreed that the ancestor or ancestors of all red algae presumably acquired a cyanobacterium as an endosymbiont that later evolved into a plastid, this hypothesis, in general terms, should not be very controversial. What is controversial is that the Cyanidiales

lineage may have acquired a thermophilic cyanobacterium as an endosymbiont that was different from the supposed primordial cyano-chloroplast that evolved eventually into all chloroplasts of red algae, green algae, and plants. If the Cyanidiales are as ancient and as separate from the main line of the red algae as proposed by Yoon et al. (2006b), there may be no overwhelming reason to reject this hypothesis.

6. Future Directions

Extensive cultivation work coupled with the application of molecular tools has begun to shed considerable light on the Cyanidiales. From only a handful of recent studies we have learned a great deal about their biodiversity at the population level, and future work that integrates genomics-level studies will no doubt greatly expand our foundational knowledge and uncover yet additional pieces of the puzzle, but it is important not to ignore phenotypic attributes, such as morphology, life history, physiology, and ecology. Current efforts that have primarily focused on populations of Cyanidiales in the geothermal features located in Italy and Yellowstone need to be expanded to consider additional geothermal islands distributed around the globe. Indeed, recent explorations of the Cyanidiales in Japan, New Zealand, and Iceland (e.g., Toplin et al., 2008) certainly indicate there is much to learn. Because of their island-like distribution, clear restrictions on transport, and relatively simple genetic structure (with no apparent sexual cycle), the Cyanidiales have become an exceptional model for addressing fundamental questions in evolution.

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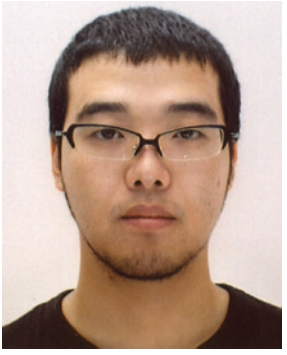

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MECHANISMS OF ACIDO-TOLERANCE AND CHARACTERISTICS OF PHOTOSYSTEMS IN AN ACIDOPHILIC AND THERMOPHILIC RED ALGA, *CYANIDIUM CALDARIUM*

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1. Introduction

Cyanidium caldarium is a primitive, unicellular red alga found in acidic hot springs throughout the world (Allen, 1959; Doemel and Brock, 1971). The alga is an obligate autotroph and has a discrete nucleus, mitochondria and a large single chloroplast but no vacuoles (Enami et al., 1975). The alga can grow at pH values as low as 0 (Allen, 1959), and the pH optimum for growth is between 1.0 and 4.0. No cell division occurs above pH 5 (Doemel and Brock, 1971). The extremely acidophilic nature of *C. caldarium* is a common feature of the Cyanidiaceae, a red algal group comprising six species, namely, *Galdieria sulphuraria, Galdieria partita, Galdieria daedala, Galdieria maxima, Cyanidium caldarium* and *Cyanidioschyzon merolae* (Albertano et al., 2000; Ciniglia et al., 2004). Among these, the complete genome of *G. sulphuraria* and *C. merolae* has been sequenced (Weber et al., 2004; Matsuzaki et al., 2004). *C. merolae* is distinguished from others by the lack of a rigid cell wall, which enables its cells to be broken down simply by freeze-thawing. *Galdieria* is a facultative heterotroph, which contrasts to the other two groups *Cyanidium* and *Cyanidioschyzon* that are obligate autotrophy (Weber et al., 2007). Like most of other species of Cyanidiaceae (Weber et al., 2007), *C. caldarium* is moderately thermophilic (grows at temperatures around 42–45°C). This makes its proteins rather thermostable, which facilitates its use as a model organism for studies on photosynthetic pigment–protein complexes of red algae. Here, we describe the mechanisms of acido-tolerance in *C. caldarium* based on studies carried out in our group as well as by other researchers. We also describe the characteristics of the two photosystems, PSI and PSII, of *C. caldarium*, and compare them with those of cyanobacteria, other eukaryotic algae and higher plants.

2. Mechanisms of Acido-tolerance in *Cyanidium caldarium*

In spite of the extremely acidic environment, the intracellular pH of *Cyanidium* cells is considered to be neutral from the following indirect lines of evidence. (1) Photosynthetic oxygen evolution of cell-free preparations of the alga, supplied

with phenyl-*p*-benzoquinone as electron acceptor, is optimal at pH 7 and inactive at pH 3 (Enami and Fukuda, 1975). (2) Most of the soluble proteins of *Cyanidium* are as acid labile as proteins of ordinary, non-acidophilic algae (Enami, 1978). (3) When *Cyanidium* cells were disrupted by a French pressure cell in distilled water, alkalization of the cell suspension from pH 5.9 to 6.5 was observed (Enami and Fukuda, 1975). These facts suggest that, in spite of the extracellular low pH environment, the intracellular pH of *Cyanidium* is maintained neutral. Thus, *Cyanidium* is expected to have a mechanism to keep the intracellular pH neutral against the steep pH gradient across the plasma membrane.

2.1. ACTIVE H^+ EFFLUX AGAINST THE STEEP pH GRADIENT ACROSS THE PLASMA MEMBRANE

Active H^+ efflux, depending on the supply of oxygen or light, was found to be indispensable for *Cyanidium* to maintain a neutral intracellular pH (Kura-Hotta and Enami, 1981, 1984). When the pH of the cell suspension was measured using a glass electrode, alkalization of the medium (an indication for H^+ influx into the cell) was observed at acidic pH in the dark when respiratory activity was inhibited by adding respiratory poisons (e.g., rotenone or antimycin) or by introducing pure nitrogen. The extent of H^+ influx increased as the pH of the medium decreased (Kura-Hotta and Enami, 1984). This indicates that H^+ leak passively into the cell according to the steep pH gradient across the plasma membrane when there is no energy supply available. The pH of the medium, previously alkalized under anaerobic conditions, returned to the initial level upon re-aeration of the cell suspension, suggesting that active H^+ efflux becomes functional again. Excess H^+, accumulated in the cell during anaerobic incubation, can now be pumped out.

Such a dependence of active H^+ efflux has not only been found for oxygen supply but also for light (Kura-Hotta and Enami, 1981). Given an acidic environment, illumination of cells caused a significant pH decrease of the medium, indicating H^+ efflux from the cells. Both, rate and extent of H^+ efflux from the cells increased as the pH of the medium was lowered, suggesting an increased activity of proton pumps at lower pH. The H^+ efflux was not affected by addition of DCMU, an inhibitor of photosystem II (PSII) electron transfer. Also, its action spectrum corresponded with the absorption spectrum of chlorophyll *a* but not with that of phycocyanin (Kura-Hotta and Enami, 1981), indicating that the light-induced H^+ efflux is driven by photosystem I (PSI).

The light-induced H^+ efflux was revealed to be an active H^+ transport system depending on intracellular ATP produced by cyclic photophosphorylation via PSI (Enami and Kura-Hotta, 1984). Triton X-100 was found to act as an effective uncoupler in intact *Cyanidium* cells without breaking down the steep pH gradient across the plasma membrane (Enami and Fukuda, 1977). Triton X-100 significantly reduced the intracellular ATP levels, stimulated the oxygen-evolving activity with phenyl-*p*-benzoquinone as electron acceptor, and completely inhibited the light-induced H^+

efflux (Enami and Kura-Hotta, 1984). Inhibition of the light-induced H^+ efflux by Triton X-100 correlated well with the depression of light-induced ATP synthesis. Furthermore, the light-induced H^+ efflux was completely inhibited by diethylstilbestrol, a specific inhibitor of plasma membrane ATPase, without any changes in the intracellular ATP level (Enami and Kura-Hotta, 1984). This indicates that the plasma membrane ATPase is responsible for active H^+ efflux in *C. caldarium*.

On the basis of the results described above, it was concluded that the active H^+ efflux is functioning to maintain the intracellular pH constant against passive H^+ influx according to the steep pH gradient across the plasma membrane. This active H^+ efflux is driven by the plasma membrane ATPase of *C. caldarium*, utilizing intracellular ATP produced by oxidative phosphorylation during respiration and cyclic photo-phosphorylation via PSI (Kura-Hotta and Enami, 1981, 1984; Enami and Kura-Hotta, 1984).

2.2. MEASUREMENT OF INTRACELLULAR pH IN *CYANIDIUM* CELLS BY ^{31}P-NMR

The conclusion stated above, is based on the observation of the external pH changes of the cell suspension. However, it is important to also determine the intracellular pH of *Cyanidium* cells under various conditions to confirm a passive H^+ influx and active H^+ efflux across the plasma membrane. Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR), developed by Moon and Richards (1973), is a powerful technique to measure the intracellular pH of intact cells, and has been applied to a variety of cells such as higher plants (Roberts et al., 1981), *Rhodopseudomonas sphaeroides* G1C (Akutsu et al., 1986), *Synechococcus* strain Y-7c-s (Kallas and Dahlquist, 1981), and *Chlorella vulgaris* (Mitsumori and Ito, 1984).

Thus, we determined the intracellular pH of *Cyanidium* cells as a function of external pH by ^{31}P-NMR (Enami et al., 1986). Figure 1 shows the ^{31}P-NMR spectra of *Cyanidium* cells incubated at external pH of 3.0 under aerobic (1) and anaerobic (2) conditions at 40°C for 1 h in the dark. Note that the three peaks derived from ATP clearly appeared in cells cultivated under aerobic conditions but almost disappeared under anaerobic conditions. The chemical shift value of inorganic phosphate (Pi) was increased by 0.89 ppm upon anaerobic treatment. The chemical shift of Pi signals caused by pH was used to calculate the intracellular pH of *Cyanidium* as a function of external pH. Cells incubated under aerobic conditions in the dark or anaerobic conditions in the light were found to maintain their intracellular pH within a narrow range (pH 6.8–7.0) even when the external pH was changed from pH 1.2 to 8.4. In contrast, the intracellular pH of cells incubated under anaerobic conditions in the dark was acidified as the external pH decreased. Upon either re-aeration or illumination of cells, the intracellular pH returned to the neutral range. In accordance with these results, the intensities of NMR signals for intracellular ATP decreased in cells incubated under dark anaerobic conditions where the intracellular pH was acidified due to

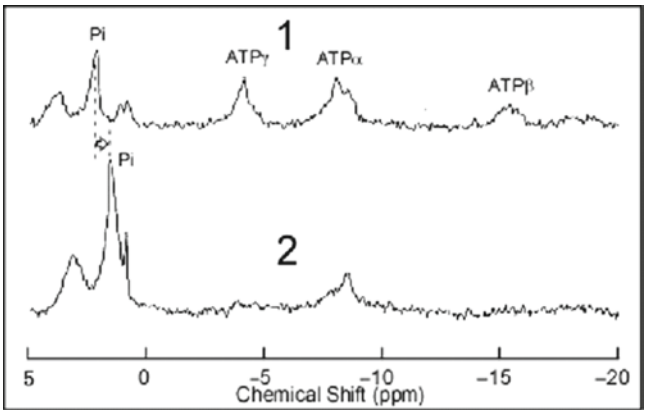

Figure 1. ^{31}P-NMR spectra of *Cyanidium* cells incubated under aerobic (1) or anaerobic (2) condition.

a lack of energy supply. On the other hand, intracellular ATP was maintained at a high level in cells incubated under aerobic conditions in the dark as well as in the light or under anaerobic conditions in the light, that is, under conditions where the intracellular pH was kept constant (Enami et al., 1986). These results indicate that when ATP production by either respiration or photosynthesis is suppressed, passive transport of protons into the cells results in an intracellular acidification. If ATP production is functional, cells are able to pump out protons and maintain their intracellular pH at a constant, physiological range.

2.3. CHARACTERIZATION OF PLASMA MEMBRANE H^+-ATPase IN *CYANIDIUM* CELLS

As described above, the intracellular pH of *Cyanidium* is considered to be maintained neutral by active H^+ efflux mediated by a plasma membrane H^+-ATPase. We have cloned and sequenced the corresponding gene, encoding the plasma membrane H^+-ATPase from *C. caldarium* (Ohta et al., 1997). The ORF comprises 2,865 bp and encodes a polypeptide of 955 amino acids with a predicted molecular mass of 105,371 Da. The deduced amino acid sequence was more homologous to those of plasma membrane H^+-ATPases from higher plants (86.5% similarity and 54.1% identity) than to that from the salt-tolerant green alga *Dunaliella bioculata* (75.1% similarity and 40.1% identity). The C-terminal region of plasma membrane H^+-ATPase from higher plants has been reported to serve as an autoinhibitory domain, important for regulation of its activity (Wolf et al., 1995). A homologous sequence, corresponding to the autoinhibitory domain, however, was not found in the C-terminal region of the plasma membrane H^+-ATPase from *Cyanidium*. This may be a characteristic feature of the red algal plasma membrane H^+-ATPase as it has the distinctive function to pump out H^+ against the steep pH gradient across the

plasma membrane. A homologous plasma membrane H^+-ATPase (98.5% similarity and 90.1% identity) was found in another acidophilic and thermophilic primitive red alga, *Cyanidioschyzon merolae* 10D, which also did not contain the regulatory/auto-inhibitory domain (Matsuzaki et al., 2004). Like *Cyanidium*, *Cyanidioschyzon* also inhabits acidic hot springs.

In preliminary experiments, we found ATP hydrolysis in cell-free preparations of *Cyanidium.* This enzyme activity exhibited typical characteristics of a plasma membrane H^+-ATPase; namely, a pH optimum around pH 6 and specific inhibition by vanadate, a specific inhibitor of plasma membrane H^+-ATPase (I. Enami, K. Kurita, 1997, unpublished data). Furthermore, the Michaelis constant (Km) for ATP hydrolysis was found to be significantly varied by pH: The $Km_{(ATP)}$ value was about 1 mM at pH 6 and about 12 mM at pH 7, indicating that the affinity of the enzyme for ATP differed over tenfold between pH 6 and pH 7. We estimated that the intracellular ATP concentration in *Cyanidium* is about 1 mM (I. Enami, M. Kura-Hotta, 1978, unpublished data) and it is therefore likely that the plasma membrane H^+-ATPase is responsible for active H^+ efflux at pH 6, but does not function at pH 7. Thus, the intracellular pH is maintained constant around pH 7 via regulation of the substrate affinity of plasma membrane H^+-ATPase (I. Enami, K. Kurita, 1997, unpublished data).

2.4. MECHANISMS OF ACIDO-TOLERANCE IN *CYANIDIUM* CELLS

In order to interpret the results described above, a model for the mechanisms of acido-tolerance in *Cyanidium* cells is illustrated in Fig. 2.

In *Cyanidium* cells grown in extremely acidic environments, H^+ passively leak into the cells due to the steep pH gradient. The H^+ leaked into the cells are pumped out against the steep pH gradient by a plasma membrane ATPase to maintain the intracellular pH in a physiological range. The plasma membrane ATPase of *Cyanidium* and other acidophilic organisms are thus unique in that they have a strong activity in the low pH region. Introduction of the gene encoding the plasma membrane ATPase from *Cyanidium* or *Cyanidioschyzon* into ordinary organisms may yield novel acido-tolerant organisms that are resistant to acid rain or can survive under acidic environments. This may provide a useful approach to overcome the environmental acidification caused by increasing pollutions.

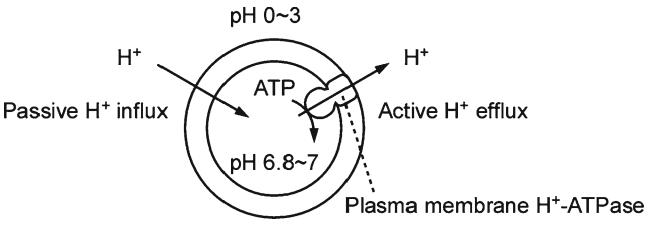

Figure 2. Schematic representation for the mechanism of acido-tolerance in *Cyanidium* cells.

3. Characteristics of Photosystems in *Cyanidium caldarium*

The photosynthetic apparatus of *C. caldarium* is essentially the same as that of other red algae (*Rhodophyta*), which appears to represent a transitional state between cyanobacteria and photosynthetic eukaryotes. The chloroplast of red algae, one of the most primitive groups of eukaryotic algae, is considered to have arisen from a single endosymbiotic event of a primitive cyanobacterial cell with a eukaryotic host. The ultrastructure of red algal chloroplasts is similar to that of cyanobacteria, where thylakoid membranes are not differentiated into stacked and unstacked membrane regions as found in chloroplasts of higher plants and green algae. Both, cyanobacteria and red algae do not synthesize chlorophyll (Chl) *b* and contain phycobilisomes as the light-harvesting antenna for the PSII complex instead of Chl *a*/*b* (or Chl *a*/*c*)-binding proteins found in higher plants and other eukaryotic algae. On the other hand, PSI of red algae has an intrinsic light-harvesting complex (LHCI) (Wolfe et al., 1994) similar to that found in all other major groups of photosynthetic eukaryotes. Thus, the red algal PSII is closely related to prokaryotic cyanobacteria, whereas its PSI is more similar to that of eukaryotic photosynthetic organisms. In the following, we focus on the characteristics of the two photosystems, PSI and PSII, of *C. caldarium*, and compare them with those of cyanobacteria and other eukaryotic algae and higher plants.

3.1. PHOTOSYSTEM II

PSII catalyzes the light-induced electron transfer from water to plastoquinone, leading to the evolution of molecular oxygen which is essential for oxygenic life on earth. The core part of protein subunits – which exists predominantly in a dimeric form – and the function of PSII are essentially the same among cyanobacteria, red algae, and higher plants. The red algal PSII resembles cyanobacterial PSII in that both contain phycobilisomes as the light-harvesting antenna and a similar set of extrinsic proteins required for oxygen evolution.

The crystal structure of PSII (Fig. 3) has been solved for two species of thermophilic cyanobacteria (Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005). Based on the crystal structure, two subunits, D1 and D2, which constitute the reaction center of PSII, are located in the center of PSII and have five trans-membrane helices each. These two subunits are surrounded by two chlorophyll *a*-binding proteins CP47 and CP43, which have six trans-membrane helices each. In addition, a large number of small, membrane-spanning subunits are located in conjunction with these four large subunits. In cyanobacteria, the number of these small subunits amounts to 13, namely, PsbE, F, H, I, J, K, L, M, Tc, X, Y, Z, and Ycf12 (Loll et al., 2005; Kashino et al., 2007; Shen et al., 2008). Genes corresponding to all of these subunits have been found in the genome of the red alga *Cyanidioschyzon merolae* (Matsuzaki et al., 2004), a close relative of *C. caldarium*. At the lumenal side, there are three hydrophilic, peripheral subunits that function to maintain the oxygen-evolving

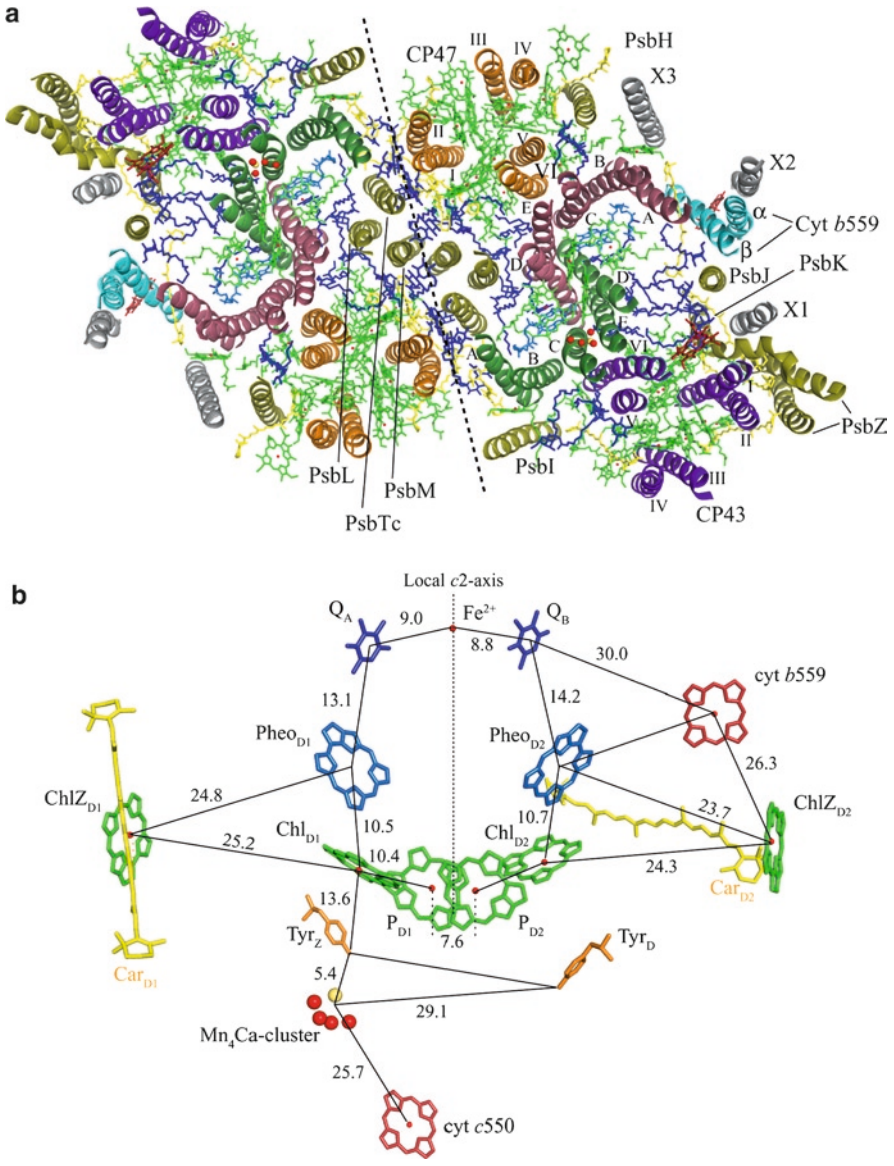

Figure 3. (**a**) Crystal structure of PSII dimer at 3.0 Å resolution (Loll et al., 2005; PDB code: 2axt). Top view from the stromal side. Color code: deep green, D1; dark purple, D2, orange, CP47; purple, CP43.; light green, chlorophylls; blue, lipids; dark yellow, carotenoids. Other subunits are indicated in the figure. For clarity, the extrinsic proteins as well as the hydrophilic loops of intrinsic subunits at the lumenal side were omitted. (**b**) The arrangement of electron transfer chain of PSII. Side view along the membrane plane. The numbers represent distances in Å.

activity. In the case of cyanobacteria, these three extrinsic proteins are the 33 kDa protein (PsbO), cytochrome c_{550} (PsbV), and 12 kDa protein (PsbU) (Shen and Inoue, 1993). These proteins were also found in *C. caldarium* PSII (Enami et al., 1998) (see below).

The PSII core complex contains 35 chlorophylls (Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005) (Fig. 3a), among which six are associated with D1 and D2 subunits, and the remaining chlorophylls are associated with CP47 and CP43, which are thus designated as intrinsic antenna subunits of PSII. The initial charge separation takes place in the reaction center chlorophyll(s) of PSII (designated as P680) upon capture of light, which subsequently transfers an electron to a pheophytin molecule bound to D1 (Fig. 3b). The electron is then transferred to the first bound plastoquinone acceptor Q_A on D2, and then to the second quinone acceptor Q_B on D1. Q_B acts as a two-electron gate which remains bound to D1 after one-electron reduction, but is released from its binding-site upon reduction by two electrons, which is then replaced by a free plastoquinone present in the plastoquinone pool of the thylakoid membrane. On the other hand, the oxidized reaction center chlorophyll abstracts an electron from a nearby tyrosine residue, namely, D1-Tyr 161 (designated as Tyr_Z), which in turn abstracts an electron from a Mn_4Ca-cluster containing four manganese atoms and one calcium atom at the donor side. Upon subsequent abstraction of four electrons, the Mn_4Ca-cluster is able to oxidize two molecules of water, leading to the release of one molecule of oxygen. This reaction thus proceeds stepwise, the intermediates of which have been designated as Si-states with i = 0–4 (Kok et al., 1970; Joliot, 2003). The initial, dark-stable state is S_1, and S_4 is a transient state immediately prior to the release of oxygen. In the crystal structure, a similar arrangement of electron transfer cofactors is found in both D1 and D2 subunits, but the active electron transfer pathway is located on the D1 side due to the presence of the Mn_4Ca-cluster on the D1 side only.

None of the PSII structures from eukaryotic organisms has been solved. Nevertheless, PSII of *C. caldarium* has been isolated to a high purity (Enami et al., 1995), and characterized in detail (Enami et al., 1998, 2000, 2003). In Fig. 4a, the protein composition of *C. caldarium* PSII is shown in comparison with that of the thermophilic cyanobacterium *Thermosynechococcus vulcanus*. As mentioned above, most of the protein subunits of PSII in cyanobacteria and red algae (and also higher plants) are similar, although the electrophoretic mobility of some subunits slightly differs for the cyanobacterial PSII and *C. caldarium* PSII. However, there is an important difference in the composition of extrinsic proteins: the *C. caldarium* PSII contains an extrinsic protein of 20 kDa (PsbQ') in addition to the three subunits homologous to cyanobacterial PsbO, PsbV, and PsbU (Enami et al., 1995, 1998, 2000, 2003; Ohta et al., 2003) (Fig. 4a, b). This 20 kDa protein has been shown to be required for effective binding of the other two proteins, PsbV and PsbU, in the red algal PSII (Enami et al., 1998). In contrast, PsbV (cytochrome c_{550}) of cyanobacterial PSII is able to bind and function independent of other extrinsic proteins (Shen and Inoue, 1993; Shen et al., 1995). Interestingly,

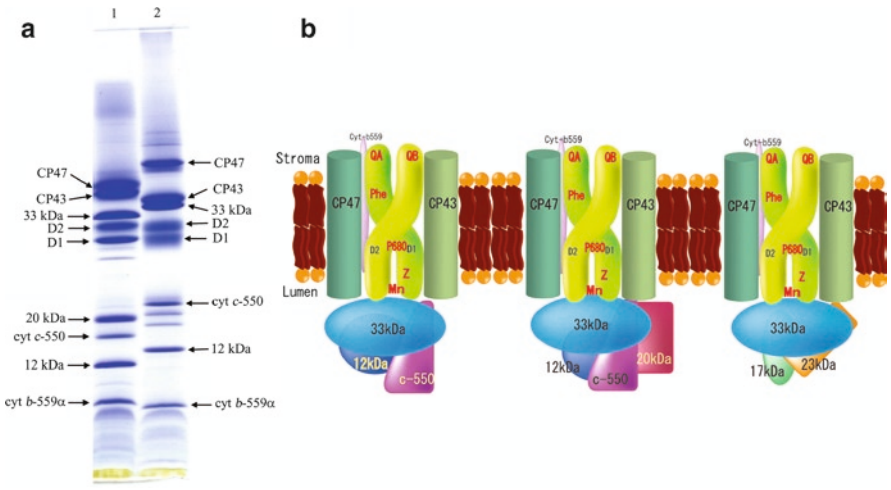

Figure 4. **(a)** Protein composition of purified PSII from *Cyanidium caldarium* (lane 1) and the thermophilic cyanobacterium *Thermosynechococcus vulcanus* (lane 2). **(b)** Schematic representation of PSII from cyanobacteria, red algae and higher plants, showing the differences of extrinsic proteins associated at the lumenal side and involved in oxygen evolution.

PsbQ' from *C. caldarium* has a low homology with PsbQ (17 kDa protein) from green algae (Ohta et al., 2003). Like higher plants, green algae contain PsbP (23 kDa protein) and PsbQ as extrinsic proteins instead of PsbV and PsbU found in cyanobacteria. Thus, the extrinsic proteins of the red algal PSII appear to represent an intermediate state between cyanobacteria and higher plants (Fig. 4b). In relation to this, it should be pointed out that PsbP-like and PsbQ-like proteins have been found in cyanobacterial thylakoids and PSII complexes (Kashino et al., 2002). The specific binding of PsbQ-like protein to cyanobacterial PSII was suggested after co-purification of PSII with a His-tagged PsbQ-like protein (Roose et al., 2007). However, cyanobacterial PSII used for crystallization, did not contain PsbQ- and PsbP-like proteins. The association of these two proteins with cyanobacterial PSII is thus not as tight as that in red algae or higher plants. Moreover, both proteins have been suggested to be lipoproteins and not required for the binding of PsbV and PsbU in the cyanobacterial PSII. Thus, the binding characteristics and function of these extrinsic proteins in the cyanobacterial PSII are apparently different from that of PsbQ' in the red algal PSII.

3.2. PHOTOSYSTEM I

PSI mediates light-induced electron transfer from plastocyanin or cytochrome c_{553} to ferredoxin, thereby generating electrons required for the reduction of $NADP^+$, which in turn is utilized for the reduction of CO_2 to organic carbon. The core part

of PSI contains 11–14 subunits denoted from PsaA to PsaO. They exhibit a high overall similarity in cyanobacteria, algae, and higher plants, although PsaG and PsaH are found only in eukaryotic PSI but not in cyanobacterial PSI (Xu et al., 2001; Scheller et al., 2001). PSI of eukaryotic algae and higher plants contains a cluster of light-harvesting proteins (LHCI, encoded by Lhca genes) that are not present in cyanobacterial PSI. Red algal PSI also contains an intrinsic chlorophyll *a*-binding antenna (Wolfe et al., 1994) encoded by Lhcr genes homologous to the Lhca genes found in green algae and higher plants (Jansson et al., 1999). Moreover, like green algae and higher plants, *C. caldarium* PSI exists in a monomeric form (Gardian et al., 2007), whereas the cyanobacterial PSI exists predominately in a trimeric form (Jordan et al., 2001). Thus, red algal PSI is more similar to that of green algae and higher plants than to that of cyanobacteria. In Fig. 5a, the protein composition of PSI from *C. caldarium* is compared with that of the cyanobacterial PSI from *T. vulcanus*.

The crystal structure of PSI from both cyanobacteria and higher plants has been reported (Jordan et al., 2001; Amunts et al., 2007). Figure 5b shows the crystal structure of PSI from pea at a 3.4 Å resolution (Amunts et al., 2007). The core part of pea PSI contains 13 subunits, namely, PsaA, B, C, D, E, F, G, H, I, J, K, L, and N. In addition, 4 LHCI subunits designated LHC1-4, are associated in the periphery of the complex. This gives rise to an asymmetric conformation of the complex, as all four LHCI subunits are located in one side of the complex. The opposite side is occupied by PsaL and PsaH, of which, PsaH has been suggested to serve as a docking site for mobile LHCII possibly migrated from PSII upon state-transitions (Takahashi et al., 2006; Amunts et al., 2007). This subunit is not present in cyanobacterial as well as red algal PSI, since the antennae of PSII in both organisms are membrane-peripheral phycobilisomes instead of LHCII. Another subunit, PsaG, has been suggested to be important for the association of LHCI with PSI core, and is also not present in the cyanobacterial PSI. The gene of this subunit was also not found in the genome of *C. merolae* (Matsuzaki et al., 2004), suggesting that the association of LHCI with PSI core in red algae may somewhat be different from that in higher plants. In relation to this it might be interesting to note that PSI from higher plants contains four Lhca proteins, whereas five Lhcr genes have been identified in the thermoacidophilic red alga (*Cyanidiaceae*) *Galdieria sulphuraria* (Marquardt et al., 2001), and the genome of *C. merolae* contains only three homologous Lhcr genes (Matsuzaki et al., 2004). The exact number of LHCI subunits of *C. caldarium* still needs to be clarified in the future.

A significant difference between PSI and PSII is the number of chlorophylls associated: PSI core binds approximately 100 chlorophylls, most of which are associated with the PSI reaction center subunits PsaA, PasB, and each LHC1 subunit binds 14 chlorophylls (Amunts et al., 2007). In total, PSI–LHCI complex of higher plants has around 160 chlorophylls; this number is likely to be similar in red algal PSI. This is in sharp contrast to the 35 chlorophylls found in the PSII core from cyanobacteria to red algae and higher plants (Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005). This low number of chlorophylls in the PSII core is

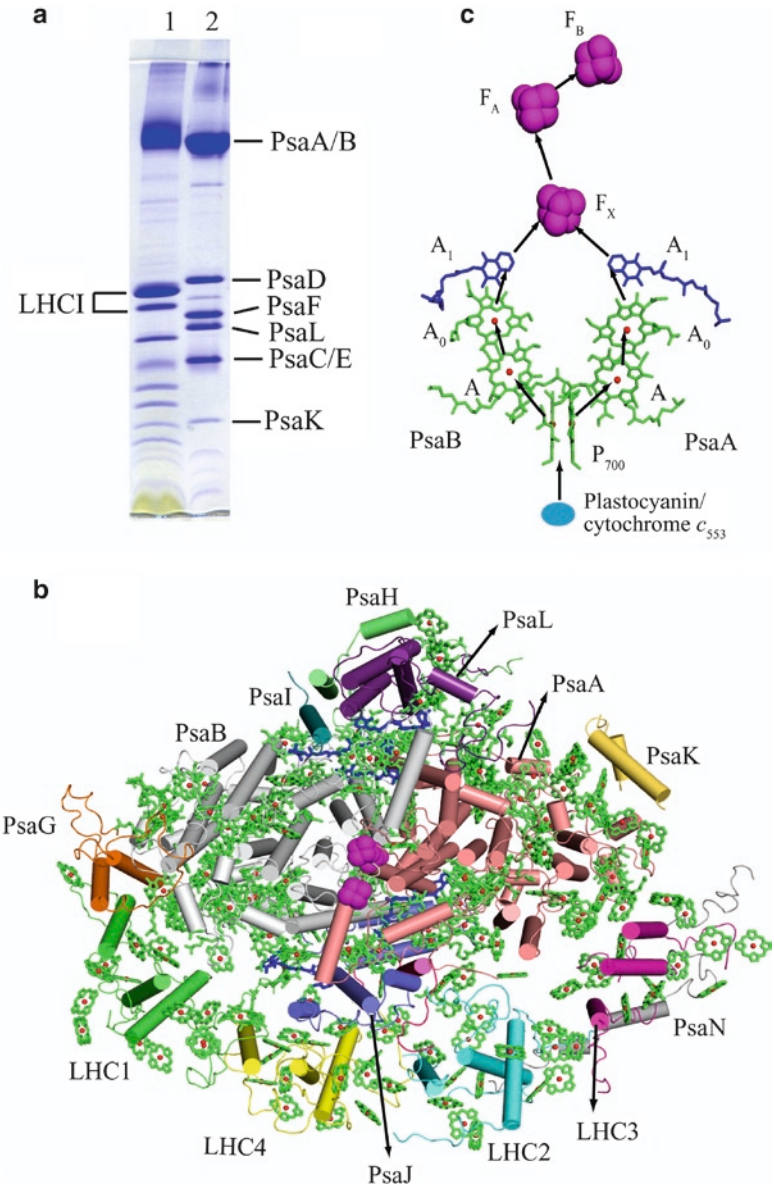

Figure 5. **(a)** Protein composition of PSI purified from *Cyanidium caldarium* (lane 1), in comparison with cyanobacterial PSI from *Thermosynechococcus vulcanus* (lane 2). (**b**) The structure of PSI monomer from pea at 3.4 Å resolution (Amunts et al., 2007; PDB code: 2oO1). Top view from the stromal side of the thylakoid membrane. For clarity, the PsaC, D, E subunits at the stromal side were omitted, and PsaF is scarcely visible due to its position at the lumenal side. Molecules in green represent chlorophylls, those in blue represent carotenoids and phylloquinones, and those in magenta represent iron-sulfur centers. (**c**) The electron transfer chain of PSI based on the crystal structure shown in **b**.

compensated by the large number of chlorophylls associated with LHCII in green algae and higher plants (typically 200–250 chlorophylls), or phycobili-proteins in cyanobacteria and red algae.

The central electron transfer chain of PSI is very similar from cyanobacteria to red algae and higher plants. The reaction center of PSI, designated P700, is composed of a chlorophyll dimer bound to PsaA and PsaB separated by a center-to-center distance of 3.6 Å (Jordan et al., 2001; Amunts et al., 2007) (Fig. 5c). This distance is significantly shorter than the distance found in the purple bacterial reaction center (7.4–7.6 Å) (Deisenhofer et al., 1995) or PSII (7.6 Å) (Loll et al., 2005), suggesting a stronger interaction between the two chlorophylls in the PSI reaction center. P700 accepts an electron from plastocyanin or cytochrome c_{553} at the lumenal side in green algae and higher plants, whereas only cytochrome c_{553} is present and denotes electrons to P700 in cyanobacteria and red alga. The electron from P700 is transferred to a chlorophyll monomer A_0, and subsequently to a phylloquinone acceptor A_1, both of which are bound to PsaA/B. The electron from A_1 is transferred to Fx, an interpolypeptide 4Fe-4S cluster located at the stromal surface of the membrane, and, subsequently to F_A, F_B, two 4Fe-4S clusters bound to the extrinsic subunit PsaC at the stromal side. The components of PSI electron transfer chain are arranged in two quasisymmetrical branches (Fig. 5c) formed by the polypeptides PsaA and B, which are homologous to each other. This suggested that both branches may be active in light-induced electron transfer (Nelson and Yocum, 2006). In the case of cyanobacteria, however, site-directed mutagenesis combined with spectroscopic studies has indicated that most of the electron transfer takes place on the PsaA-branch. In contrast, studies on algal and higher plant PSI have yielded evidence for a significant activity on the PsaB-branch. Whether both branches are active or only one branch is active in the red algal PSI needs to be clarified in future studies.

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Biodata of **Renate Scheibe**, author (with **Nicolas König**) of ***"Redox-Modification of Chloroplast Enzymes in Galdieria sulphuraria: Trial-And-Error in Evolution or Perfect Adaptation to Extreme Conditions?"***

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REDOX-MODIFICATION OF CHLOROPLAST ENZYMES IN *GALDIERIA SULPHURARIA*: TRIAL-AND-ERROR IN EVOLUTION OR PERFECT ADAPTATION TO EXTREME CONDITIONS?

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1. Introduction

Regulation of enzyme activities is required for metabolism, in particular for assimilatory pathways in plastids of all photosynthetic eukaryotic organisms as well as in prokaryotes performing oxygenic photosynthesis. In order to be able to adjust the metabolic fluxes to the actual energy input and the demand, various enzymes have developed structures that are suitable for post-translational regulation by covalent redox-modification (Dietz et al., 2002). Reversible reduction/oxidation of cysteine residues is extremely suited for this purpose. It is mediated by thioredoxins that are present in all organisms (for review see: Buchanan, 1980).

Since reduction and reoxidation take place simultaneously, and these interconversions are fine-tuned by metabolites acting as positive or negative effectors, the steady-state level of active enzyme molecules can be easily changed and thus enzyme activities are adjusted to the actual requirements (Scheibe, 1991; Faske et al., 1995). When no reductant from photosynthesis is available, all enzyme molecules will be oxidized. In this case, redox-modulation functions as a switch to avoid futile cycling of reductive and oxidative pathways. The Calvin-cycle enzymes NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (NADP-GAPDH), fructose 1,6-bisphosphatase (FBPase), sedoheptulose 1,7-bisphosphatase (SBPase), and phosphoribulokinase (PRK) are inactive upon oxidation, and the oxidative pentose-phosphate pathway with glucose 6-phosphate dehydrogenase (G6PDH) as redox-modulated key enzyme is activated upon oxidation. In addition, various other chloroplast enzymes, such as NADP-dependent malate dehydrogenase (NADP-MDH), ATPase, ADP-glucose pyrophosphorylase (AGPase), and RubisCO activase, are also redox-regulated (for review see Buchanan and Balmer, 2005). In general, however, the regulatory properties of these enzymes have been described mainly for higher plants and for green algae. Much less information is available for other photoautotrophic organisms, but in some cases the origin of the plastidial enzymes can be traced back to cyanobacteria (Martin et al., 1999).

This review will focus on some of these enzymes (Fig. 1) from the rhodophyte *Galdieria sulphuraria* and compare their properties and enzymes with those from higher plants. The phylogenetic origin and the position of this organism in

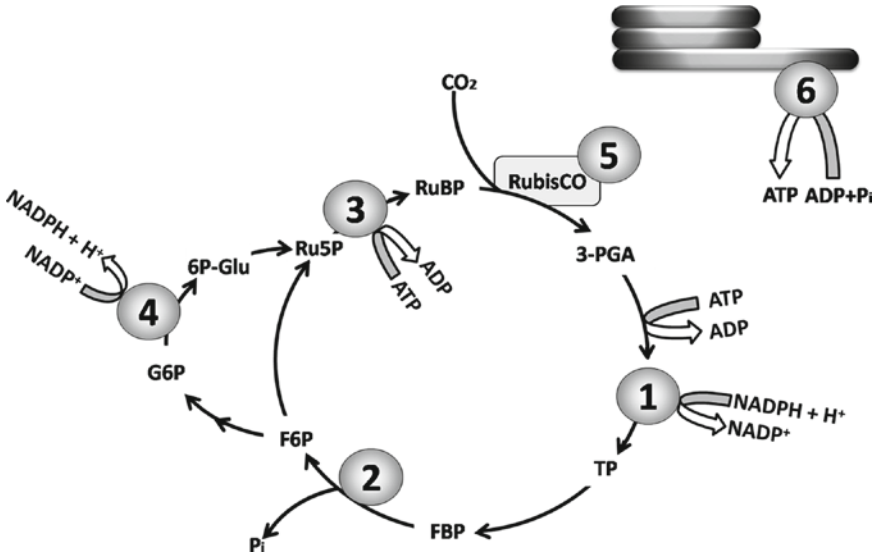

Figure 1. Redox-modulated chloroplast enzymes: The enzymes that are subject of this article are highlighted in the scheme by numbers. 1: NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (NADP-GAPDH), 2: fructose 1,6-bisphosphatase (FBPase), 3: phosphoribulokinase (PRK), 4: glucose 6-phosphate dehydrogenase (G6PDH), 5: RubisCO activase (RCA), 6: ATPase, γ-subunit.

the evolutionary tree of life are still under debate (Reyes-Prieto et al., 2007). Therefore, the differences between higher plant chloroplast enzymes and the red algae enzymes can be seen either as a snapshot on the pathway of trial and error in evolution, or as a perfect adaptation to the specific environmental and metabolic conditions encountered by this thermoacidophilic organism.

G. sulphuraria is exceptional in its property not only to grow photoautotrophically in extreme environments, but also mixo- and heterotrophically, allowing for survival under unfavorable conditions (Gross and Schnarrenberger, 1995). Therefore, the differences in the regulation of some enzymes of basic metabolism that are summarized in this review will also have to be discussed in the light of the environments of this extremophile.

2. Regulation of NADP-Dependent Glyceraldehyde 3-Phosphate Dehydrogenase (NADP-GAPDH) and Phosphoribulokinase (PRK)

NAD(P)-dependent GAPDH catalyzes the reductive step of CO_2 assimilation in the Calvin cycle using 1,3-bisphosphoglycerate (1,3-bis-PGA) as a substrate. In higher plants, starting with multicellular green algae (Charophyceae), this enzyme occurs as a heterotetramer consisting of subunits A and B, where GapB is unique for the higher plant lineage. GapB is very similar to GapA, but is characterized by a

C-terminal sequence elongation with two cysteine residues involved in redox modification. The active reduced A_2B_2 form, which consists of both subunits is subject to oligomerization upon oxidation leading to the inactive A_8B_8 form (Baalmann et al., 1995). *In vivo*, light (reductant) and 1,3-bis-PGA are required for dissociation and activation (Baalmann et al., 1994). Due to the lack of GapB in unicellular red and green algae, inactivation of the active A_4 form of these organisms is achieved by the formation of a complex with PRK and the small chloroplast protein CP12 (Graciet et al., 2003). The inactive form of this complex is easily activated and dissociated by a reductant. Both complexes, A_8B_8 and the bienzyme complex with CP12, are present in higher plant chloroplasts (Scheibe et al., 2002), thus enabling a two-step activation of GAPDH, where 50% of the GAPDH and 100% of PRK are easily released in their active form simply with a weak reductant or in low light. The capacity of GAPDH can be increased when an increasing 1,3-bis-PGA level signals the requirement for higher fluxes through this step.

The linker protein CP12 appears to be present in all autotrophic prokaryotic and eukaryotic organisms, in most cases consisting of the two characteristic sequence loops formed due to the presence of two cysteine pairs (Pohlmeyer et al., 1996; Wedel and Soll, 1998). Some cyanobacteria, glaucophytes, and the red algae, however, possess CP12 proteins with one of these cysteine pairs or a single cysteine lacking (Wedel et al., 1997). In the Galdieria protein, the two N-terminal cysteines are lacking, and the amino acid sequence in this part does not exhibit any similarity with the higher plant CP12 proteins (Fig. 2) (Oesterhelt et al., 2007a). This CP12 protein, however, is still capable of forming the oxidized GAPDH/PRK/CP12 complex, but PRK activity in this complex remains almost as high as it is in the reduced dissociated form. GAPDH activity in the complex, on the other hand, is decreased by more than 50% (Oesterhelt et al., 2007a). Since the N-terminal part of the higher plant CP12 sequence has a high similarity with the extra C-terminus of GapB, and provides the binding domain for GAPDH in the complex, it is surprising to observe a functional inactivation of GAPDH in the Galdieria complex, although the cysteines thought to form the loop structure in the oxidized CP12 for binding GAPDH are absent.

PRK in Galdieria, in contrast, is equipped with the two regulatory cysteines, as known from the higher plant enzyme (Porter et al., 1988), but with seven amino acids less between these cysteines (Fig. 3). It is, however, only marginally affected in its activity when it is part of the complex (Oesterhelt et al., 2007a).

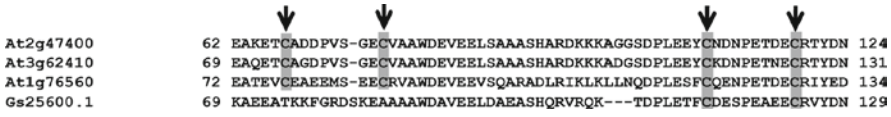

Figure 2. Sequence comparison of CP12 isoforms from *A. thaliana* with the sequence deduced from the *G. sulphuraria* gene (Gs25600.1). The Cys residues involved in the loop formation upon oxidation are indicated by arrows.

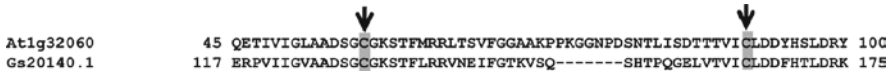

Figure 3. Sequence comparison of the N-terminal part of PRK from *A. thaliana* with the sequence deduced from the *G. sulphuraria* gene (Gs20140.1). The cysteine residues found to be redox-active in the higher plant enzyme are marked with arrows.

3. Regulation of Chloroplast Fructose 1,6-bisphosphatase (FBPase)

Plastid FBPase, catalyzing the first irreversible step of the Calvin cycle, is also subject to redox-modification in higher plants. Upon reduction its affinities towards the substrate FBP and towards Mg^{2+} are increased and the pH optimum is shifted to less alkaline values. On the other hand, the oxidized form is essentially inactive under physiological conditions (Schürmann and Wolosiuk, 1978). When a heterologously expressed FBPase from *G. sulphuraria* was analyzed at pH 8, there was only a twofold increase of enzyme activity upon reduction. In addition, the affinity towards Mg^{2+} and the pH optimum of the red algal enzyme were not significantly changed upon reduction (Reichert et al., 2003). This finding is rather surprising, since a similar redox-modulation as in the higher plant enzyme was expected due to the presence of the two cysteine residues (155 and 174 in the spinach FBPase) involved in the redox change in the higher plant enzyme (Reichert et al., 2003). Inspection of the amino acid residues between these regulatory cysteines, however, makes it likely that additional features are required for efficient redox-modulation of FBPase activity that are not present in the *G. sulphuraria* enzyme (Fig. 4). It seems that in the red algal enzyme the disulfide bridge between the two conserved cysteines is not formed, possibly due to the lack of an arginine residue in front of Cys 174 (Reichert et al., 2003).

An interesting feature of the extremophile *G. sulphuraria* is the presence of two copies of plastid FBPase, while the closely related *Cyanidioschizon merolae* exhibiting obligate photoautotrophy possesses only one copy, as is the case for all higher plants. One of the two copies in *G. sulphuraria* is apparently only expressed under autotrophic growth conditions, while both are expressed when sugars are present and the algae grow mixotrophically. It will be of interest, therefore, to analyze in detail the properties of the two red algal plastid FBPases, since a gene duplication might have produced closely related isoforms with distinct regulatory

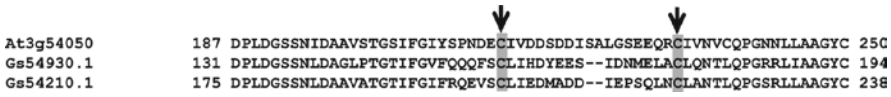

Figure 4. Comparison of the sequence insertion of the chloroplast FBPase from *A. thaliana* with the corresponding sequences those from *G. sulphuraria* (Gs54930.1 and Gs54210.1); the latter enzyme has been cloned from a cDNA library, expressed in *E. coli*, and was characterized (Reichert et al., 2003).

differences in order to accommodate growth, both with sugar production and with sugar consumption that would require active FBPase also in darkness for interconversions of sugar carbon skeletons when sugars with different carbon skeletons are taken up and used for growth. As another specific difference, a high number of genes for sugar transporters has been found to be unique for Galdieria when compared to *C. merolae* (Barbier et al., 2005).

Another phosphatase, namely sedoheptulose 1,7-bisphosphatase (SBPase) is also redox-modulated in higher plants, but possesses a completely different motif with two conserved cysteines in the N-terminal part (Dunford et al., 1998). In the Galdieria genome, a sequence (Gs51920.1) that exhibits 45% identity to the SBPase sequence from *A. thaliana* (At3g55800) was identified. Interestingly, two of the six cysteines of the Arabidopsis enzyme are conserved in the Galdieria sequence (data not shown). These cysteines (Cys52 and Cys57) had been identified as candidates for redox-regulation using site-directed mutagenesis of the Arabidopsis enzyme (Dunford et al., 1998). However, experimental evidence is not available yet, as to whether the presence of these cysteines is sufficient to obtain redox-modulation in the red algal enzyme.

4. Regulation of Glucose 6-Phosphate Dehydrogenase (G6PDH)

As a key enzyme of the oxidative pentose-phosphate pathway G6PDH is present in both compartments, plastid and cytosol of algae and plants, and also in cyanobacteria. The plastidal and the cyanobacterial enzymes are inactivated by reduction and consequently are only active in their oxidized forms as they occur in darkness (Buchanan, 1991; von Schaewen et al., 1995). Although an endosymbiotic origin of chloroplasts can be assumed, the cyanobacterial G6PDH has a completely different set of redox-active cysteines in its sequence, but is already subject to light/dark modulation via thioredoxins (Udvardy et al., 1984). Red and green algae as well as plants possess a plastidial G6PDH with a new set cysteines, that are responsible for its light/dark modulation (Wenderoth et al., 1997). While there are four plastidial isoforms in *A. thaliana* (Wakao and Benning, 2005), *G. sulphuraria* is equipped with one gene coding for a plastidic isoform, containing the sequence motif with two conserved cysteines in positions as in the higher plant enzymes (Fig. 5). By comparison

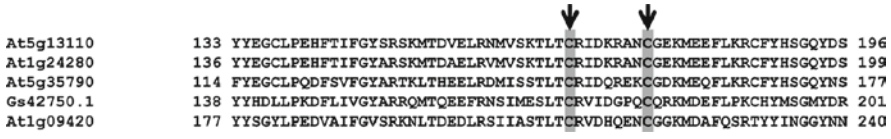

Figure 5. Sequence comparison of the chloroplast isoforms of G6PDH from *A. thaliana* with the corresponding sequence from *G. sulphuraria* (Gs42750.1).

with the 3D-structure of the Leuconostoc enzyme it was shown that these cysteines are located in an exposed region of the NADP-binding domain (Wenderoth et al., 1997). The second gene coding for G6PDH in Galdieria is apparently cytosolic, because it does not carry a transit sequence. Its amino acid sequence corresponding to the redox-active region in the plastid isoform, however, is characterized by cysteine residues in similar positions (data not shown).

5. Other Redox-Modulated Enzymes

In higher plants, various further enzymes have been found to be redox-regulated. However, the structure motifs that are responsible for redox-dependent activation or inactivation are not known in all cases. Here we will highlight some of the enzymes that are closely related to CO_2 assimilation.

5.1. CHLOROPLAST ATP-SYNTHASE (F_0F_1-ATPase, COUPLING FACTOR CF_1)

It is long known that the γ-subunit of the coupling factor in chloroplast thylakoids is responsive to redox-changes (Mills and Mitchell, 1982). The redox-active cysteine residues are located on an insert of nine amino acids that is unique for the chloroplast protein in higher plants and not present in the cyanobacterial protein (Werner-Grüne et al., 1994). Such insert is absent in all sequences with reasonable similarity in the Galdieria genome (Fig. 6). The sequence most related to the higher plant sequence is shown (Gs14490.1). Interestingly, in the diatom *Odontella sinensis* the γ-subunit of chloroplast ATPase also lacks the insert with the two regulatory cysteines (Pancic and Strotmann, 1993).

5.2. RUBISCO ACTIVASE (RCA)

Entry of carbon into the Calvin cycle by fixation of CO_2 through RubisCO is a key step in all autotrophic organisms. Interestingly, RubisCO from red algae is characterized by a higher specificity factor towards CO_2 when compared to the properties of RubisCO from higher plants, green algae, and cyanobacteria (Uemura, et al., 1997). The red algal RubisCO has been crystallized and the structure analysis

Figure 6. Comparison of sequences for the γ-subunit of chloroplast ATPase from *A. thaliana* with a similar gene sequence from *G. sulphuraria* (Gs14490.1).

revealed a hexadecameric L_8S_8 form as present in all other eukaryotic organisms, but the genes for both subunits are localized on the plastid genome (Baranowski and Stec, 2007).

In higher plants, a specific activase enzyme appears to be required in order to revert unproductive binding of sugars or inhibitors (Zhang and Portis, 1999). This RubisCO activase itself is redox-dependent and its larger isoform carries two cysteine residues at the C-terminus possibly involved in thioredoxin f-dependent redox modification (Zhang et al., 2001). When searching the Galdieria database for sequences that are similar to RubisCO activase, we could not get any indication for the presence of such enzyme in Galdieria. Possibly, growth of these organisms under such extreme conditions requires a high specificity, but no reductive mechanism for activation, since light might be available only scarcely.

5.3. ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase)

In red algae, floridean starch is synthesized in the cytosol by α-glucan synthases using UDPglucose, but a plastidial pathway with ADPglucose as a substrate has also been postulated for red algae (Sesma and Iglesias, 1998; Viola et al., 2001). From the Galdieria-genome project, however, no evidence was obtained for the existence of a plastidial AGPase, whereas UGPase was detected (Weber et al., 2004).

From BLAST searches using both, the small and the large subunit of *A. thaliana* AGPase, we obtained evidence that the large catalytic subunit might be present in *G. sulphuraria*, but no protein with reasonable similarity to the small regulatory subunit was found. Identities with the large subunit of AGPases or other sugar-nucleotide transferases were around 40%, but identities with small subunit sequences ranged below 10%. This is particularly interesting, since the redox-active cysteines are localized on the higher plant small subunit, allowing for the formation of an intermolecular disulphide bond (Geigenberger et al., 2005).

6. Components of the Thioredoxin System

6.1. THIOREDOXIN REDUCTASES (FTR AND NTR)

In higher plants, the main reductive pathway for redox-modulation of chloroplast enzymes in the light is the ferredoxin–thioredoxin system that delivers electrons from photosystem I via ferredoxin and a ferredoxin–thioredoxin reductase (FTR) to the various thioredoxins (for review see Schürmann and Buchanan, 2008). FTR is a unique protein consisting of two subunits, where the large conserved subunit carries both, a [4Fe-4S] cluster and a disulphide bridge. When the Galdieria genome was searched for related sequences initially no related sequence could be found among the deduced proteins. When, however, the nucleotide sequences and the six possible frames for translation were analyzed more closely, a sequence with

66% identity to a sequence from *C. merolae*, and with 54% identity to the FTRB sequence from *A. thaliana* was found be located on stig_35:141422…141721.

As an alternative, in cytosol and mitochondria NADPH is used as a reductant, and a specific reductase (NTR) catalyzes the electron transfer to the thioredoxins (see review: Jacquot et al., 2009). In addition, a novel enzyme, NTRC that consists of an NTR domain and a thioredoxin domain on one polypeptide chain, has been recently localized in the chloroplast compartment (Serrato et al., 2004). A rather high identity (68%) of a Galdieria gene (Gs37990.1) was found with the NTRA and NTRB sequence from *A. thaliana*, but also with the reductase domain of the NTRC (50%). It is, however, evident from this comparison that the putative Galdieria NTR sequence does not possess an extension with a thioredoxin domain. The gene product of Gs37990.1 does not appear to possess a transit peptide; therefore, a cytoplasmic localization has to be assumed.

6.2. THIOREDOXINS (TRX)

Higher plants possess a large variety of redox-active proteins that has been classified into several families (Meyer et al., 2005). Thioredoxins are one major group of redox-active proteins with the conserved active site WCGPC. They are ubiquitous, stable, small proteins of around 10 kDa. We have searched the Galdieria genome for similar sequences and found six sequences with rather high identities (Table 1), including the active-site motif. One of the putative thioredoxins from Galdieria (Gs46260.1) carried a varied active-site motif where the tryptophane was replaced by glycine, but otherwise a high identity with *A. thaliana* thioredoxin y2 was apparent. Three of the six sequences, namely the ones related to Trx x, y2 and f1, are chloroplast-localized in *A. thaliana*, the others have cytosolic (or nuclear) localization (Table 1). In fact, more sequences were found with obvious Trx similarity, but of much larger size, apparently belonging to protein-disulphide isomerases, which carry various Trx domains.

Table 1. Genes from *G. sulphuraria* with high identities to *A. thaliana* thioredoxins. Common name and subcellular localization of the Arabidopsis thioredoxins are also indicated.

Trx in Galdieria	MAtDB	Common name	Localization	Identity [%]
Gs13580.1	At5g42980	Trx h3	Cytosol	42.6
Gs49640.1	At3g51030	Trx h1	Cytosol	34.6
Gs45060.1	At3g17880	TDX	Cytosol/Nucleus	44.6
Gs28820.1	At1g50320	Trx x	Chloroplast	40.5
Gs44280.1	At3g02730	Trx f1	Chloroplast	40.8
Gs46260.1	At1g43560	Trx y2	Chloroplast	39.1

While in higher plants all thioredoxins are nuclear-encoded, in the red alga *Porphyra yezoensis* a chloroplast-encoded thioredoxin has been found (Reynolds et al., 1994). When the putative *G. sulphuraria* thioredoxins were aligned with this Porphyra sequence, Gs28820.1, which is most similar to thioredoxin x in *A. thaliana*, it turned out to possess 43% identity. But there is no plastid-encoded thioredoxin in *G. sulphuraria*. From sequence comparisons it seems that all those thioredoxins from Galdieria which are similar to the chloroplast-located genes in Arabidopsis possess a putative transit peptide.

6.3. FERREDOXIN (FD) AND FERREDOXIN-NADP REDUCTASE (FNR)

Electron flow from photosystem I to NADP for CO_2 assimilation requires ferredoxin and ferredoxin-NADP reductase (FNR). In addition, as found in heterotrophic tissues in plants, for assimilatory processes depending on reduced ferredoxin, NADPH from OPP activity could provide reducing equivalents via FNR (Suzuki et al., 1985). In fact, roots possess specialized isoforms of ferredoxin and of FNR to allow for reverse electron flow from NADPH, for example, for nitrite and sulphite reduction (Onda et al., 2000).

In *A. thaliana*, two leaf-type and two root-type FNRs with a rather high identity between the two related isoforms (80% between the two leaf-type FNR forms, and 77% between the two root-type FNRs) are present. The two groups show only 51–53% identity between them. Searching for genes that code for similar proteins in *G. sulphuraria*, we found Gs25860.1 with 56% identity with one of the leaf-type FNRs, namely At5g66190 (FNR1). The identity with At1g20020 (FNR2) was at 47%; and with the root-type FNR forms 43–44%. It is thus most likely that the identified *G. sulphuraria* gene codes for an FNR.

When we searched the *G. sulphuraria* genome for ferredoxin sequences, using each of the six ferredoxin sequences from *A. thaliana* (Hanke et al., 2004), one Galdieria sequence also appeared with highest scores, namely Gs40280.1. Upon alignment of all *A. thaliana* sequences with this sequence it grouped after Fd1, Fd2, and Fd3 (58–60% identity) and before Fd4, Fd5, and Fd6 (32–41% identity) when using the sequences of the mature proteins (Fig. 7). The characteristic cysteine residues involved in ligating the [2Fe-2S] cluster of all ferredoxins was also found in another *G. sulphuraria* gene, (Gs43140.1) which had 29% identity with Gs40280.1, and 30% identity with the Fd5 form *A. thaliana.* In a phylogenetic tree it becomes evident that Gs40280.1 might be positioned slightly closer to the typical root Fd3 as compared to the leaf forms Fd1 and Fd2 (Fig. 8). This might be of particular interest, since the environmental conditions of *G. sulphuraria* might require a less negative redox potential of the ferredoxin in order to allow for electron transfer from NADPH in low light or dark as is the case in root plastids. The specific motifs involved in a redox-potential shift of the FeS-cluster cannot easily be distinguished, since higher-order structures might be responsible for this property (Gou et al., 2006).

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At1g10950 Fd1      ------MASTALSSAIVSTSFLRRQQTP--ISLRSLPFAN-----TQSLPGLKSSTARGG 47
At1g60950 Fd2      ------MASTALSSAIVGTSFIRRSPAP--ISLRSLPSAN-----TQSLPGLKSGTARGG 47
At2g27510 Fd3      ------MATVRISSTSMTKAVLRSQTTNKLITNKSYNLSVGSTKRVSRSFGLKCSANSGG 54
Gs40280.1          ------------------------------------------------------------
At5g10000 Fd4      ------MDQVLYSSYIIKIPVISRISPS--QAQLTTRINN------TTYFGLSSSRGNFG 46
At4g14890 Fd6      ------MATLPLPTQTSTISLPKPYLSN----SFSFPLRN-------ATLSTTTNRRNFL 43
At1g32550 Fd5      ------MALILPCTFCTSLQKKNFPINRRYITNFRRGATTAT---CEFRIPVEVSTPSDR 51
Gs43140.1          MNGFCESRTTFHSNRNRKLTSFHHCCPLYGSSRYYTRILRRLTRNCQQYLSWTCHGETFG 60
                                                                 ↓  ↓  ↓
At1g10950 Fd1      RVTAMATYKVKFITPE--GEQEVECEEDVYVLDAAEEAGLDLPYSCRAGSCSSCAGKVVS 105
At1g60950 Fd2      RVTAMATYKVKFITPE--GELEVECDDDVYVLDAAEEAGIDLPYSCRAGSCSSCAGKVVS 105
At2g27510 Fd3      -ATMSAVYKVKLLGPD-GQEDEFEVQDDQYILDAAEEAGVDLPYSCRAGACSTCAGQIVS 112
Gs40280.1          ----MASYKIHLVNKDQGIDETIECPDDQYILDAAEEQGLDLPYSCRAGACSTCAGKLLE 56
At5g10000 Fd4      KVFAKESRKVKLISPE-GEEQEIEGNEDCCILESAENAGLELPYSCRSGTCGTCCGKLVS 105
At4g14890 Fd6      TTGRIIARAYKVVVEHDGKTTELEVEPDETILSKALDSGLDVPYDCNLGVCMTCPAKLVT 103
At1g32550 Fd5      GSLVVPSHKVTVHDRQRGVVHEFEVPEDQYILHSAESQNISLPFACRHGCCTSCAVRVKS 111
Gs43140.1          DPNAR-KYRVTVRNQRTGECYIRDVPTDRYIWWSFLEQGIELPSSCVNGCCTTCACRVVS 119
                                  ↓
At1g10950 Fd1      GSIDQSDQSFLDDEQMSEGYVLTCVAYPTSDVVIETHKEEAIM---------------- 148
At1g60950 Fd2      GSVDQSDQSFLDDEQIGEGFVLTCAAYPTSDVTIETHKEEDIV---------------- 148
At2g27510 Fd3      GNVDQSDGSFLEDSHLEKGYVLTCVAYPQSDCVIHTHKETELF---------------- 155
Gs40280.1          GQVDQSDQSFLDDDQVKAGFVLTCVAYPTCNATILTHQEESLY---------------- 99
At5g10000 Fd4      GKVDQSLGSFLEEEQIQKGYILTCIALPLEDCVVYTHKQSDLI---------------- 148
At4g14890 Fd6      GTVDQS-GGMLSDDVVERGYTLLCASYPTSDCHIKMIPEEELLSLQLATAND-------- 154
At1g32550 Fd5      GELRQPQALGISAELKSQGYALLCVGFPTSDLEVETQDEDEVYWLQFGRYFARGPIERDD 171
Gs43140.1          GRIEQPLALGLLKEMKNKRYALLCVSYPKSDVEVVLQEEDEVYCRQFGDSFESGGVEYGG 179
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Figure 7. Comparison of full-length amino-acid sequences for the ferredoxins from *A. thaliana* with those from *G. sulphuraria.*

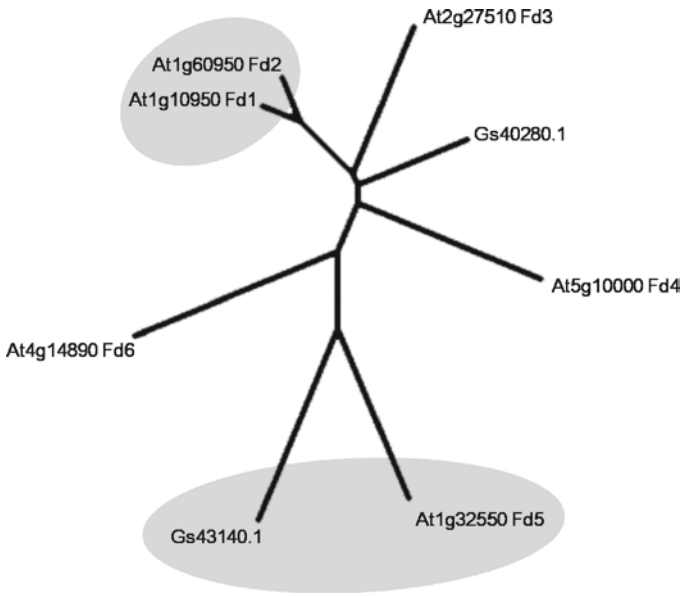

Figure 8. Unrooted phylogenetic tree of leaf and root ferredoxin amino-acid sequences from *A. thaliana* and the Fd from *G. sulphuraria* was drawn after aligning the sequences in ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2/index.html).

7. Metabolic Flexibility and Unique Regulatory Properties of Enzymes of CO_2 Assimilation in Galdieria

Galdieria occurs in hot springs around the world and can survive under extreme conditions (Ciniglia et al., 2004). It even exhibits cryptoendolithic growth in volcanic environments, where it can function in the absence of light, taking up organic compounds excreted by other organisms (Gross et al., 1998). Nowadays it is assumed that *G. sulphuraria* does not grow mixotrophically, because sugars repress photosynthetic O_2 evolution (Oesterhelt et al., 2007b). *G. sulphuraria* is unique among the Cyanidiales in being able to grow heterotrophically in the presence of a large variety of sugars and sugar alcohols (Gross and Schnarrenberger, 1995). Galdieria is characterized by the presence of unique genes coding for sugar transporters that can be induced upon a change of the growth conditions (Weber et al., 2004). Comparative genomics have revealed the different equipment with genes coding for sugar uptake and metabolism in *G. sulphuraria*, not present in a closely related, but obligate autotrophic red alga, namely *C. merolae* (Barbier et al., 2005).

The high metabolic flexibility required for growth under extreme conditions might have led also to distinct regulatory properties of enzymes normally involved only in photoautotrophic carbon assimilation. These enzymes will have to function in very low light or even darkness, and thus are equipped with less stringent or no structural features for oxidative modification. In this respect it is interesting to note that in general there seems to be a lower susceptibility to hydrogen peroxide of algal thiol-modulated enzymes than of the corresponding higher plant enzymes, as was shown for FBPase, GAPDH, and PRK of some green algae and for the cyanobacterial enzymes (Takeda et al., 1995; Tamoi et al., 1998).

8. Summary

Post-translational regulation of various chloroplast enzymes is achieved by reversible reduction/oxidation of cysteine residues often located on inserts in amino-acid sequences of otherwise highly similar isoforms of other compartments, which are not subject to such mechanisms. Redox-modification requires the simultaneous presence of reducing power and oxygen and thus could be established during evolution after the appearance of oxygenic photosynthesis. In most cases, a reductant is provided by photosynthetic electron transport from the light reactions. In higher plants and green algae, a clear separation between reductive and oxidative processes is ensured by complete shutdown of the OPP in the light through G6PDH inactivation. On the other hand, light activation of Calvin-cycle enzymes is dependent on reduced thioredoxins in addition to an increase of the metabolites to be converted. This also prevents any activity of carbon assimilation during the dark phase. In organisms related to ancient forms that developed early during evolution and which occur mainly in aqueous environments with lower light intensities and less pronounced light/dark cycles, a less strictly regulating redox-mechanism has been found.

At the same time, the sequences responsible for such regulatory mechanism, namely the positions of the cysteines in amino-acid inserts that are involved in disulphide bridge formation, are either less complex or lacking. This could be interpreted as an expression of a less advanced stage of molecular evolution that has not yet reached the stage of sophistication as found in higher plants (Table 2).

Alternatively, however, it could be the result of an adaptation to the particular environments characterized by the lack of high light intensities and fluctuations. In the case of *G. sulphuraria* it could have arisen from the unique property to grow both, photoautotrophically and heterotrophically on a large variety of sugars. In the latter situation, the carbon assimilation pathway might be used partially for interconversions of the sugar substrates that are taken up by the algal cells in order to integrate these carbon skeletons into the common pathways. In this case, the Calvin-cycle enzymes FBPase and PRK must be active also in the dark as found for Galdieria. In addition, CO_2 assimilation should proceed as soon as only little light is available. Furthermore, RubisCO activity in Galdieria appears to be independent upon regulation by an activase which in higher plants is redox-modulated. Finally, in polyglucan synthesis, which might occur in the cytoplasm, AGPase (and UGPase) is responsible for glucose activation, but no regulatory, redox-active small subunit appears to be present.

In conclusion, we have compiled work from biochemical experiments and molecular cloning with searches in the recently published complete genome of *G. sulphuraria* (http://genomics.msu.edu/galdieria/index.html) in order to draw a picture of the regulatory properties of carbon-assimilation enzymes. Using these facts, we try to relate our findings to the physiological requirements encountered by this extremophile. In future work, more physiological and biochemical experiments are required to verify some of these assumptions that have been deduced from sequence information only.

Table 2. Presence of redox-regulatory properties in target enzymes in Galdieria and in higher plants.

Target enzyme	Galdieria	Higher plants
Rubisco activase	(*enzyme not present*)	pronounced
GAPDH	moderate	pronounced[a]
FBPase	none	pronounced
SPBase	moderate	moderate
PRK	little	pronounced
CP12	minimal	pronounced
AGPase	none	pronounced
F_0F_1-ATPase	none	pronounced
G6PDH	pronounced	pronounced
NADP-MDH	(*enzyme not present*)	pronounced

[a] For GAPDH, a new type of redox-regulated isoform (namely GapB) appeared in addition.

9. Acknowledgments

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THE THERMO-ACIDOPHILIC CYANIDIOPHYCEAE (CYANIDIALES)

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1. Introduction

1.1. BACKGROUND

Extremophiles are organisms that thrive in environments previously thought inhospitable to life because the physicochemical characteristics fall outside the range tolerated by human cells. These types of environments are usually fatal to most eukaryotes, but have been shown to host a diverse group of prokaryotes that rely on specialized enzymes for survival. Extremophiles are permanently exposed to harsh environmental conditions and are categorized according to their ability to thrive in a specific type of niche. For example, thermophiles grow at temperatures above 50°C, psycrophiles prefer temperatures below 15°C, piezophiles are pressure-lovers, halophiles are found in high salt concentrations, whereas acidophiles and alkaliphiles thrive at an extreme pH of ≤ 3 and ≥ 10, respectively. These taxa are found in hot and cold deserts, hot springs, salt lakes, in sulfide mines, or near deep-sea vents all around the world. It has been speculated that if extraterrestrial life exists, it would be in the form of an extremophile.

The unicellular red algae Cyanidiales are one of the few eukaryotes that can thrive in an extreme environment. They are defined as thermo-acidophiles because they have the remarkable ability to grow in volcanic and thermal areas under extremely low pH (0.05–5) and relatively high temperature (35–56°C). Cyanidiales are the only photosynthetic eukaryotes living under such hostile conditions.

1.2. HISTORY OF CYANIDIALES

Known since the second half of the nineteenth century, these unique algae were first considered a single species and described as *Coccochloris orsiniana* by Meneghini (1839). However, due to their simple morphology and lack of diagnostic features, this alga was assigned many different names and classified in various algal divisions (Chlorophyta, Cryptophyceae, Cyanophyta, Rhodophyta) in the

ensuing years (see Ott and Seckbach, 1994 for a review). It was not until 1933 that Geitler gave it the generally accepted binomial *Cyanidium caldarium* and the taxon was officially recognized as part of the Rhodophyta by Hirose (1958), who demonstrated the presence of several characteristic rhodophycean features.

It was discovered only fairly recently that several species of thermo-acidophilic algae actually coexist in the same habitats (De Luca and Taddei, 1970), explaining the persisting confusion about the taxonomy of the Cyanidiophyceae. Thereafter, De Luca et al. (1978) introduced the new species *Cyanidioschyzon merolae*, easily recognized by its characteristic size and shape, whereas Merola et al. (1981) differentiated *Galdieria sulphuraria* from *Cyanidium caldarium* based on its ability to grow heterotrophically. In 1991, Sentsova isolated three new species of *Galdieria* from far eastern Russia: *G. daedala*, *G. maxima*, and *G. partita* that are differentiated by cell size, number of autospores produced, and plastid shape (Gross, 1999; Sentsova, 1994). Finally in 2007, Pinto et al., described a new "phylospecies" *G. phlegrea* that mainly differed from other *Galdieria* by virtue of the relative number of substitutions in the plastid-encoded *rbc*L gene. This species is found exclusively in endolithic sites in thermal areas in Italy. Until now, the Cyanidiales includes seven species. However, with the advance of molecular systematics, and the exploration of new environments, the number of recognized species of Cyanidiales is very likely to increase, perhaps dramatically.

2. Characterization of the Cyanidiales

2.1. ECOLOGY

Cyanidiales are widely distributed and, according to Brock (1978), could virtually be found in every acidic hot spring around the world as long as all ecophysiological conditions are met (Fig. 1). With the increased interest in these unique algae, many volcanic regions have been explored (Negoro, 1944; Pinto and Taddei, 1978; Geitler and Ruttner, 1936; Schwabe, 1936; Brock, 1978; Sentsova, 1991; Gross et al., 1998, 2001; Ciniglia et al., 2004) and they have been recorded from all continents with the exception of Africa and Antarctica (perhaps due to limited effort). Cyanidiales have been found to thrive not only in acidic hot pools and springs (Fig. 1a) but also on rocks and muddy soil around hot pools and along streams (Fig. 1c) and in fissures in rock walls (interlithic) or under a thin layer of rock (endolithic) that is exposed to acidic sulfur fumes (Fig. 1b).

Cyanidiales grow optimally at 45°C but can be found in environments with a temperature up to 55–56°C. The lower limit in aquatic habitats is around 35–36°C because below these temperatures, there is apparently too much competition with other acidophilic algae for their persistence. In contrast, they can be found in soil with temperatures as low as 10°C where competition is apparently less marked (Doemel and Brock, 1971). The pH range at which Cyanidiales grow is between 0.05 and 5, with the optimum at pH 2–3 (Doemel and Brock, 1971).

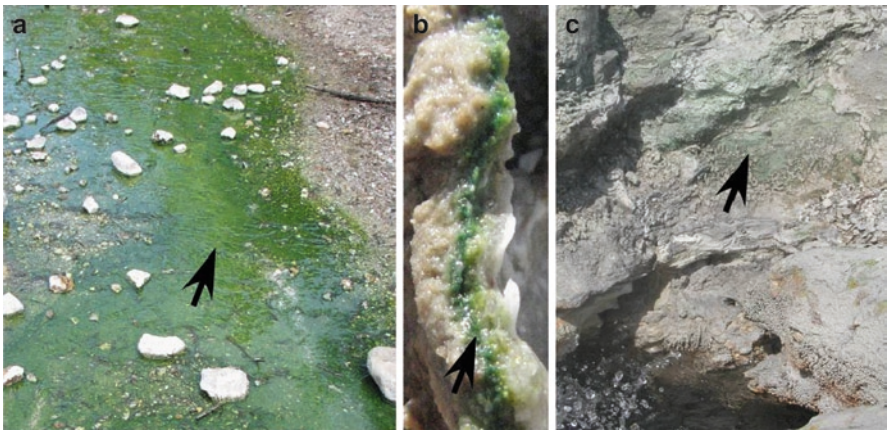

Figure 1. Images of Cyanidiales in their natural environment. (**a**) Cyanidiales growing as a thick mat in the runoff from an acidic hot spring. (**b**) Endolithic Cyanidiales growing under a crust of rock deposits. (**c**) Cyanidiales growing on a rock surface that is exposed to sulfur fumes. All of these sites are found at Yellowstone National Park in the USA.

Members of the Cyanidiophyceae have also been rarely found in nonthermal and nonacidic habitats. Schwabe (1936) was the first to record *Cyanidium* from caves along the coast of central and southern Chile. Additional "cave" Cyanidiales were recorded from France (Leclerc et al., 1983), Israel (Friedmann, 1964), and Italy (Skuja, 1970; Ciniglia et al., 2004). Caves have a specific microclimate: stable, low temperatures (4–10°C), slightly acidic to alkaline (pH 5–7), constant and high level of humidity, and low light intensity. Although these ecological features are radically different from those associated with the "typical" Cyanidiales, *Cyanidium* from caves have morphological characteristics that are virtually indistinguishable from their thermo-acidophilic counterparts (Hoffman, 1994). In fact cave taxa show a close phylogenetic relationship to *Cyanidium caldarium* based on molecular data (Ciniglia et al., 2004). During their survey of 120 acidic sites in Italy, Pinto and Taddei (1978) documented the presence of *C. caldarium* not only from hot springs, but also nonthermal sites such as sulfur mines where sulfidic ores lead to the establishment of low pH conditions. *Galdieria* strains were recorded together with chlorophytes and Cyanobacteria from a sulfur mine, a peat bog, and diatomite shield exposed to oxygen at Soos in Czech Republic (Gross et al., 2002). Finally, Cyanidiales have been reported in freshwater contaminated by acid mine drainage (AMD) that displays extremely low pH and high metal concentrations (Moreira et al., 1994; Gross, and Gross, 2001; Aguilera et al., 2007; Baker et al., 2004). The extremely low pH of these waters is due to the dissolution and oxidation of sulfidic minerals (sulfides) in rock strata that are exposed to water and oxygen (Novis and Harding, 2007). In turn, the low pH facilitates metal solubility in water, and therefore AMD waters tend

to have high concentrations of heavy metals (Johnson, 1998). It is possible that a greater diversity of red algae may be present in mine environments than has been identified thus far. For instance, Moreira et al. (1994) reported at least three strains of *Galdieria* from the Rio Tinto (Spain). Currently, there are no available nucleotide sequences of Cyanidiales from mines and their phylogenetic placement is still unknown. Therefore, these intriguing environments deserve more attention in the future. Until now, there have been no reports of Cyanidiales from basic freshwater or seawater habitats (Brock, 1978; Gross, 1999).

Cyanidiales also have the ability to grow under low light conditions, for instance in endolithic conditions where the algae that are covered by a silica sinter receive less than 1% of daylight for photosynthesis, or within caves up to 40 m from the entrance where direct light reaches the algal colonies only rarely (Gross et al., 1998; Leclerc et al., 1983). Growth in crypto-endolithic habitats or caves is most likely a strategy to avoid desiccation, but requires special adaptations for the organism to survive under reduced light intensity. Autotrophic growth at endolithic sites is possible only within the first few millimeters beneath the surface layer (Gross and Oesterhelt, 1999). Therefore, the ability of *Galdieria* to grow heterotrophically in the dark offers a great advantage to invade such ecological niches. In this particular case, the organic substances (i.e., sugars) necessary for heterotrophic growth are released by dead cells in the lower regions of the algal layer (Gross et al., 1998). Another mode of adaptation demonstrated by Cyanidiales in these low-light habitats is an increase in photopigment concentration within the plastid.

All thermo-acidophilic environments are inhabited by a mixed population of Cyanidiales but different species dominate at different sites (Ciniglia et al., 2004; Yoon et al., 2006a; Pinto et al., 2007). The dominance of one species seems to be driven by the difference in humidity rather than by temperature or pH variation between habitats (Pinto et al., 2007; Ciniglia et al., 2004; Yoon et al., 2006a; Doemel and Brock, 1971). For instance, *Cyanidioschyzon* clearly dominates the most humid environments (e.g., springs and pools) and both *Galdieria* and *Cyanidium* thrive in relatively dry habitats, whereas only *Galdieria* is present in dry endolithic habitats (Ciniglia et al., 2004; Pinto et al., 2007). These results are confirmed by laboratory work in which *G. sulphuraria* was found to be more resistant to water loss than *C. caldarium* (Pinto et al., 2007). The ability of *Galdieria* to grow heterotrophically on a unique spectrum of substrates and its preference for extremely low pH apparently creates an ecological niche that is not accessible to other algae.

2.2. MORPHOLOGY

There are clearly defined ecophysiological, cytomorphological, and biochemical differences between the three Cyanidiales genera (Merola et al., 1981; Seckbach, 1991, 1994, 1999; Pinto et al., 1994; Albertano et al., 2000). *Cyanidioschyzon* has

oval to club-like shape and reproduces by binary fission, whereas *Cyanidium* and *Galdieria* are spherical and differ in average cell size and number of endospores produced per sporangium. Other cytomorphological differences include the lack of a cell wall for *C. merolae*, differences in chloroplast and mitochondrion shape, absence of dictyosomes for *C. merolae*, and absence of vacuoles in *C. merolae* and *C. caldarium* (for review, see Albertano et al., 2000).

C. caldarium and *C. merolae* are able to utilize both nitrate and ammonium as a nitrogen source, but are strict autotrophs. In contrast, *Galdieria* species grow only on ammonium, tolerate higher salt concentrations, and can also grow heterotrophically on several carbon substrates. Nevertheless, some of the ecophysiological differences seem to be strain-specific, and depend in most part on the temperature at which these algae were tested (for review, see Albertano et al., 2000).

3. Taxonomy and Molecular Phylogeny of Cyanidiales

Morphological and physiological features often fail to distinguish the Cyanidiales from other unicellular rhodophytes. Only a few characters, for example, the absence of a pyrenoid, a character regarded as nonprimitive (Ueda, 1994), distinguish this group of acidophilic unicells from other "primitive" (i.e., bangiophyte) red algae (Broadwater and Scott, 1994).

Recent molecular data confirm that the Cyanidiales are evolutionary distantly related to other unicellular algae (Bhattacharya and Medlin, 1995; Cozzolino et al., 2000). Based on a multigene phylogeny using plastid genes, Yoon et al. (2006b) formally recognized the order Cyanidiales within the Rhodophyta and placed it in its own subphylum Cyanidiophytina, class Cyanidiophyceae. This subphylum defines the most ancient split within the Rhodophyta that was estimated to have occurred ca. 1.3 million years ago (Yoon et al., 2004). These algae were once thought to be one of the first photosynthetic eukaryotes to have evolved on our planet, representing the missing link between cyanobacteria and the Rhodophyta (Seckbach, 1987, 1994, 1999). More recent molecular data show that Glaucophytes are the most anciently diverged photosynthetic organisms (Reyes-Prieto and Bhattacharya, 2007). However, red algae played a key role in algal evolution by contributing the plastid to the supergroup Chromalveolata via secondary endosymbiosis (e.g., Yoon et al., 2002).

Currently, phylogenetic analyses support the division of the Cyanidiales into four distinct lineages (Ciniglia et al., 2004; Yoon et al., 2004, 2006a; Fig. 2). The first is the *Galdieria* lineage (excluding *G. maxima*) that is divided into the *Galdieria* A (*G. sulphuraria, G. daedala,* and *G. partita*) and *Galdieria* B (*G. phlegrea*) clades. *Galdieria* A includes strains from worldwide locations and the order of branching within this clade follows a clear geographical pattern. *Galdieria* B consists of taxa from dry and endolithic habitats exclusively from Italy. These two clades do not have distinguishing morphological features but appear to differ with respect to ecophysiology. *Galdieria* B has a maximum growth rate at a lower

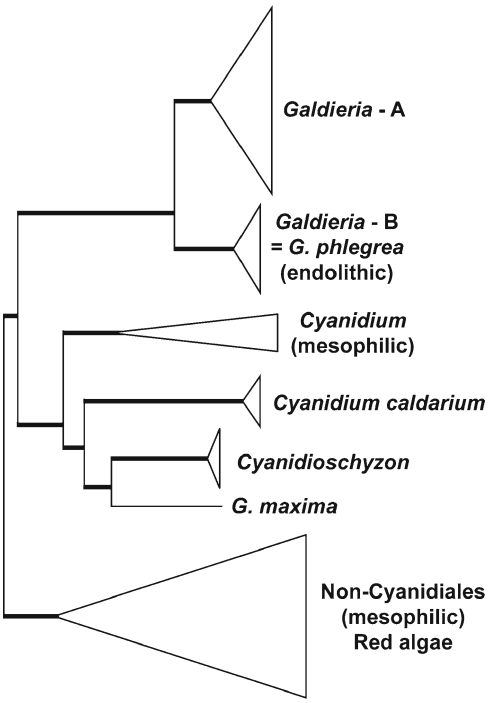

Figure 2. Schematic representation of the Cyanidiales phylogeny showing the four major lineages and their interrelationships. The thick branches denote well supported monophyletic groups according to Ciniglia et al. (2004), Yoon et al. (2004), and Yoon et al. (2006).

temperature (ca. 25°C) and a lower value for the maximum photosynthetic rate compared to other *Galdieria*, thereby suggesting that members of this clade are likely shade-adapted (Pinto et al., 2007). The second major lineage is represented by *Cyanidium caldarium* that has been sampled worldwide. In contrast to *Galdieria* A, the *Cyanidium caldarium* clade does not show a phylogenetic branching pattern consistent with geographical separation. This may indicate that these taxa are very successful at dispersal (i.e., panmictic). Sister to the *Cyanidium caldarium* lineage is the mesophilic *Cyanidium* spp. that forms a novel monophyletic group. Members of this group have been found in nonacidic and nonthermal habitats in caves in Italy. It is very likely that strains found in caves in other parts of the world or in mines will group with the Italian specimens. Finally, the fourth lineage includes *Cyanidioschyzon merolae* and *G. maxima*. This sister group relationship is quite intriguing because *Cyanidioschyzon* is morphologically distinct from other Cyanidiales by virtue of the absence of a cell wall and vacuole and division through binary fission. As in *Cyanidium caldarium*, the phylogenetic structure of the *Cyanidioschyzon* clade is discordant with their geographical distribution suggesting a panmictic population. Molecular (i.e., solely *rbc*L) data suggests that

there is greater Cyanidiales diversity in nature that remains to be discovered and that this diversity is unlikely to be reflected in their morphological and ecophysiological characters.

Phylogenetic relationships within the Cyanidiales were resolved with high confidence using 16S rRNA and five plastid genes (Yoon et al., 2004). This work revealed that the *Galdieria* clade was the first to diverge followed by the mesophilic *Cyanidium* clade, followed finally by the sister lineages *Cyanidioschyzon* (including *G. maxima*) and *Cyanidium caldarium*. Given these results, it was suggested that the ancestral state for Cyanidiales was thermo-acidophily and that mesophily is a derived state. The Cyanidiales apparently trace their origin to extremophilic ancestors that thrived during the early volcanic phase of our planet's evolution. However, the red algal ancestor was likely to have been mesophilic as found for early diverging extant members of its sister groups the chlorophyte and glaucophyte algae.

4. Mechanisms of Dispersal and Establishment of New Population

Very little is known about dispersal mechanisms of the Cyanidiales. Although these algae are scattered throughout the world, their habitats are usually very small and locally restricted to thermal and/or acidic sites. Both physical (e.g., lower temperature, dryness) and chemical (e.g., pH closer to neutrality) barriers separate individual hot springs and their communities of organisms from each other. In addition, the Cyanidiales are known to have reduced tolerance to desiccation, which potentially renders them incapable of crossing mesophilic habitats and undergoing long-distance migration (Smith and Brock, 1973; Brock, 1978; Gross, 1999). Laboratory experiments with *Galdieria* show that a decrease to 95% humidity could cause a 10,000-fold reduction in cell viability (Smith and Brock, 1973), whereas a relative humidity level below 85%, or 4 days dehydration at 40°C could be lethal (Gross et al., 2002; Pinto et al., 2007). Moreover, Cyanidiales apparently fail to form resting spores to allow survival during prolonged periods of desiccation. Consequently, Cyanidiales are expected to undergo rapid interpopulation divergence due to geographic isolation, leading to the establishment of species or races. This seems to be the case on a global scale for the genus *Galdieria*. A molecular phylogeny including strains from different regions of the world shows a clear grouping according to a geographical pattern (Ciniglia et al., 2004; Yoon et al., 2006a).

However, dispersal does occur, and environmental barriers to mobility do not appear to be an absolute hindrance to spread. In contrast to *Galdieria*, *rbc*L phylogenies suggest that identical or closely related strains of *Cyanidium* and *Cyanidioschyzon* can be found in distantly located hot springs regardless of their geographic origin (Ciniglia et al., 2004; Yoon et al., 2006a). These genera seem to be subject to constant gene flow between world populations, promoted by an unidentified dispersal mechanism.

Another clue to the dispersal of Cyanidiales is their ability to successfully colonize new thermal areas that constantly arise anew due to volcanic activity. For example, hot springs are of a limited lifespan with an individual vent in Yellowstone National Park (Mammoth area) existing from days or weeks to several years (Bonheyo et al., 2005). The eruption of a new hot spring creates an environment suitable for colonization by thermophilic organisms.

How thermophiles reach new environments or how widely separated the source population must be to deter dispersal remains unknown. Brock (1967) suggested that extant thermophilic microorganisms may be relict populations from times when volcanic habitats dominated the Earth, implying that long-distance dispersal is not necessary to explain worldwide distribution. This could be illustrated by the apparent lack of Cyanidiales on the relatively recent Hawaiian Islands. However, because these islands are located in the middle of the Pacific Ocean, it is likely that they were simply not yet subject to a founder event (Brock, 1978). In addition, this hypothesis does not explain the presence of Cyanidiales in Iceland or the Azores (Gross et al., 2001; Gross and Oesterhelt, 1999; Brock, 1978; Castenholz, 1969), islands that emerged from the ocean due to the volcanic activity of the mid-Atlantic Ridge with no previous physical contact with other landmasses.

Worldwide dispersal of the Cyanidiales is probably not the result of one unique mode or mechanism of dispersal, but rather a combination of several strategies. These dispersal modes are likely to be passive because Cyanidiales do not have flagellae for self-propulsion or gliding ability (Gross, 2000). The most obvious dispersal mechanism is by direct contact between close springs via a fluid medium such as spring runoff. However, even in close contact, if the physical and chemical conditions in each spring are not met, dispersal will not occur. For example, Fouke et al. (2003) found little similarity in bacterial community composition between five travertines at Angel Terrace (Yellowstone National Park) despite the rapid and continuous flow of spring water between the travertines. Direct contact between hot springs can also occur through subsurface hydrothermal connections. But here again, connections do not exist between springs of distantly located hydrothermal areas, and even within a particular hydrothermal region such as Yellowstone, springs can be hydraulically isolated (White et al., 1988) and underground water temperatures might be too high for organisms to survive.

At Yellowstone National Park, Brock (1994) observed that elks and bison often cross the thermal areas in search of warmth during the winter, and insects such as Ephydrid flies feed on cyanobacterial mats in hot springs. In spite of this, thermal areas remain mostly inhospitable to wildlife in general and it is unlikely that dispersal via an animal host is responsible for long-distance dispersal of the Cyanidiales. The most viable mechanism to explain the global distribution of thermophiles is via hydrothermal vapor from volcanic areas. Bonheyo et al. (2005) showed that thermophiles become aerosolized and that updrafts of steam from a hot spring may transport thermophilic bacteria, albeit in low numbers. It is more likely that long-range dispersal is the result of major volcanic activities where vapors can be effectively propelled into the air and displaced by winds.

5. The Cyanidiales as a Model to Understand Life in Extreme Environments

The Cyanidiales are particularly interesting organisms to study at the genomic level because they have adapted to extreme environments under high selection pressures that have existed for hundreds of millions of years. Unlike most eukaryotes, they are capable of tolerating a large array of toxic chemical compounds such as sulfuric acid, arsenic, and other heavy metals, and thrive at a pH and temperature that are lethal to most eukaryotes. Until now, the genome of two Cyanidiales have been sequenced: *Cyanidioschyzon merolae* (Matsuzaki et al., 2004; Nozaki et al., 2007) and *Galdieria sulphuraria* (Barbier et al., 2005a). Compared to other photosynthetic organisms, *C. merolae* appears to have unusual and simple genome characteristics. These characteristics may reflect primitive features that were extensively modified during the evolution of other algal and plant lineages, or represent specific adaptations to the extreme environment (Nozaki et al., 2007). Data from other Cyanidiales genomes are necessary to differentiate between these two hypotheses.

Genome comparison between related organisms such as *C. merolae* and *G. sulphuraria* is key to unraveling the molecular basis of the observed structural, physiological, and metabolic differences. Both taxa belong to the same lineage, but diverged a long time ago (Yoon et al., 2004; Gross et al., 2001) and show fundamental differences such as the absence of a cell wall and vacuole in *C. merola* or differences in the mode of replication. The most remarkable physiological difference is probably the metabolic versatility of *Galdieria sulphuraria* that can grow heterotrophically and mixotrophically on more than 50 different carbon sources (Rigano et al., 1976, 1977; Gross and Schnarrenberger, 1995; Gross, 1999; Oesterhelt et al., 1999). In addition, *Galdieria* has the ability to withstand higher toxic concentrations of heavy metals (Albertano and Pinto, 1986; Nagasaka et al., 2004) and high concentration of salt (Gross et al., 2002) than does *Cyanidioschyzon*.

5.1. *CYANIDIOSCHYZON* GENOME

The nuclear genome sequence of the unicellular *Cyanidioschyzon merolae* 10D was completed in 2004 and is the first completed algal genome (Matsuzaki et al., 2004). With a size of only 16.5 Mbp (20 chromosomes), it is one of the smallest genome among all photosynthetic eukaryotes. A total of 5,331 genes have been identified, of which at least 86% are expressed. The genome shows a relatively low degree of genetic redundancy and only 26 genes (0.5%) contain introns. It contains the smallest set of rRNA genes with three copies at three different loci, giving rise to a single small nucleolus without nucleolus-associated chromatin. Because *C. merolae* is simple in structure and gene composition, it provides an excellent model to study the origin and evolution of photosynthetic eukaryotes, as well as the fundamental mechanisms essential for the survival of free-living eukaryotic cells.

5.2. *GALDIERIA* GENOME

The *Galdieria sulphuraria* 074G genome project was initiated in 2004 (Barbier et al., 2005b). The final genome size was estimated to be approximately 15 Mbp. Using pulsed-field gel electrophoresis, the number of chromosomes was estimated to be approximately 42 (Weber et al., 2007b), in contrast to previous studies that reported 2–40 chromosomes (Muravenko et al., 2001; Moreira et al., 1994). Approximately 50% of the *Galdieria* genes contain one or several short introns (45–60 bp) displaying typical spliceosomal features (Barbier et al., 2005b). Intergenic regions are also reported to be small in this taxon.

5.3. GENOME COMPARISONS

As of 2005, 70% of the *G. sulphuraria* genome was sequenced (Barbier et al., 2005b) and ca. 3,000 ESTs are available for this taxon (Weber et al., 2004). Barbier et al. (2005) compared these data to the *C. merolae* genome. Several major findings came out of their comparison, some of which could explain the structural and physiological differences between the two lineages.

5.3.1. Genome Similarities

More than 30% of *G. sulphuraria* genes did not have orthologs in the *C. merolae* genome. This difference is in agreement with the fact that both organisms are deeply diverged within red algae and have adapted to different extreme environments.

5.3.2. Membrane Transporters

The photoautotroph *C. merolae* exhibits a strikingly similar enzymatic spectrum involved in carbohydrate metabolism as does the heterotrophic and mixotrophic *G. sulphuraria*. Therefore, it is not the lack of enzymes involved in carbohydrate metabolism that constrains *C. merolae* to obligate photoautotrophy. However, the *Galdieria* genome encodes a larger number of putative carbohydrate transporters in comparison to the *C. merolae* genome, thus implying that these transporters might play a key role in the metabolic flexibility of this taxon. These transporters are likely responsible for the uptake of various carbon sources (Oesterhelt et al., 1999; Oesterhelt and Gross, 2002) that are subsequently used for heterotrophic growth (Gross and Schnarrenberger, 1995). Without these uptake systems for sugars, *C. merolae* must rely exclusively on photosynthetic activity for survival.

5.3.3. Tolerance to Heavy Metal

G. sulphuraria harbors cation transporters that are absent from the *C. merolae* genome. These transporters might be involved in the extraordinary tolerance of *Galdieria* to toxic metal ions.

5.3.4. Cell Wall Biosynthesis

Because *C. merolae* lacks a cell wall, it is not expected to encode enzymes involved in cell wall biosynthesis, modification, or degradation. Barbier et al. (2005a) found several putative enzymes unique to *G. sulphuraria* that may be involved in metabolizing complex cell wall polysaccharides.

5.3.5. Adaptation to Osmotic Stress

Floridoside and isofloridoside are major photosynthetic products found in all red algae with the exception of the order Ceramiales (Kirst, 1980; Nagashima, 1976; Karsten et al., 1999). The presence of both floridoside and isofloridoside has been reported in members of the Cyanidiales, however with less certainty in *Cyanidioschyzon* for which only trace amounts have been recorded (De Luca and Moretti, 1983; Nagashima and Fukuda, 1983; Reed, 1983). Although the floridoside biosynthetic pathway is known at the biochemical level, nothing is known about the genes encoding the enzymes involved in this biosynthesis. The comparison of genome sequences and ESTs data from *G. sulphuraria* led to the identification of several candidate genes that might be involved in floridoside biosynthesis. None of the most promising candidates were found in the *C. merolae* genome (Barbier et al., 2005a).

Floridoside has several known functions within a cell, some of which might explain why *Galdieria sulphuraria* often thrives at endolithic habitats where both desiccation and high salinity are likely to affect cell function, whereas *C. merolae* is restricted to osmotically stable habitats (Gross et al., 1998; Gross and Oesterhelt, 1999; Ciniglia et al., 2004; Yoon et al., 2006a). According to Barbier et al. (2005a), the reduced capacity of *C. merolae* for osmotic adaptation is likely due to the absence of vacuoles, a cell wall, and inability to synthesize osmolytes, the latter two being connected to floridoside metabolism. Floridoside is a small compatible solute (or specific type of osmolyte) that is used by cells of numerous organisms under water-stress to maintain cell volume by having a protective effect on macromolecules such as proteins and enzymes. For example, high salt concentration and desiccation both result in an increase in floridoside concentration in different red algae (Kerjean et al., 2007; Karsten et al., 1993; Kauss, 1968; Reed, 1985; Reed et al., 1980).

Floridoside has also been suggested as a direct precursor of polysaccharides in the cell wall of the red algae *Porphyridium* (Li et al., 2001, 2002), and could be partly responsible for the wall-less *Cyanidioschyzon*. However, other genes missing from the *Cyanidioschyzon* genome may be responsible for the absence of a cell wall. For example, genes encoding enzymes that might be involved in the cell wall synthesis (e.g., fucosyltransferases, galactosyltransferase, xylanase) were found exclusively in the *G. sulphuraria* genome (Barbier et al., 2005a). In addition, Class III peroxidases usually found associated with the cell wall (Li et al., 1989) and typical occurring in terrestrial microalgae were identified only in the *Galdieria* genome, thus supporting the key function of these enzymes in the process of land colonization (Oesterhelt et al., 2007).

6. Future Applications

Whereas most organisms encode a set of conserved functions evolved for life in a mesophilic habitat, ancient extremophiles such as Cyanidiales offer a fascinating window into cell and genome evolution in a wide range of harsh physical and chemical conditions. Comparative genomic analyses of extremophiles with other organisms can provide valuable information about biochemical and evolutionary strategies that push the physical and chemical limits of life. This approach can also lead to the identification of enzymes or other biological products with potential biotechnological applications (e.g., *Taq* polymerase). The study of extremophiles will provide a better understanding of the origin of life and evolution of key mechanisms involved in cell function. In this regard, the study of extremophiles has been of particular interest to astrobiologists in search of life beyond Earth. By understanding how life can survive or thrive under the most extreme range of physical and chemical conditions on Earth, scientists hope to be able to predict how life might have been established and flourished in habitats beyond our planet. For example, endolithic environments on Mars and other planets constitute potential refugia for extraterrestrial microbial communities.

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CHILEAN CAVE *CYANIDIUM*

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1. Introduction

Many cave ecosystems on Earth function with bacteria at the base of the trophic chain (Northup and Lavoie, 2001, Sarbu et al., 1996). Although much is known about cave heterotrophic microorganisms, (Barton and Northup, 2007; Northup and Lavoie, 2001) less is known about photosynthetic microorganisms like cave algae and cyanobacteria (Albertano and Urzi, 1999). Taxonomic surveys of lamp associated flora, algae and cyanobacteria, in electrically lit passages within show caves have been reported (Faimon et al., 2003; Smith and Olson, 2007). Other groups have reported bioactive metabolites isolated from cyanobacteria found in caves, but without further details on the description or the ecology of the microorganisms (Antonopoulou et al., 2002, 2005). A similar trend is found for unicellular algae, as is the case of the Cyanidiales (Seckbach, 1994b), a group of asexual, unicellular red algae usually found in acidic (pH 0.5–4.0), high temperature (40–56°C) sites around hot springs (Doemel and Brock, 1970). Actually, most of these algae are blue-green, due to the pigments c-phycocyanin and chlorophyll-a (Toplin et al., 2008).

The Cyanidiales are classified into three genera, *Cyanidium*, *Cyanidioschyzon*, and *Galdieria* (Seckbach, 1994a; Ciniglia et al., 2004; Saunders and Hommersand, 2004; Yoon et al., 2006), of which *Cyanidium caldarium* is the most studied member. This alga is characterized by rounded cells with thick walls, a nucleus, a single, often large chloroplast, a vacuole, and one mitochondrion (Ford, 1984; Ott and Seckbach, 1994; Albertano et al., 2000). *Cyanidium* cells reproduce by internal divisions forming four daughter cells or autospores inside the mother cells (Ford, 1984).

Both the 16S rRNA (Walker et al., 2005; Yin et al., 2008), the *rbcL* and *psaA*/*B* genes codified in the chloroplast (Ciniglia et al., 2004), as well as the 18S rRNA in the chromosomal DNA (Smith and Olson, 2007) had been used for phylogenetic classification and identification in environmental samples. These phylogenetic studies suggest that the Cyanidiales are one of the most ancient groups of algae, having diverged about 1.3 billion years ago at the base of the Rhodophyta (Glöckner et al., 2000; Seckbach, 1994b; Yoon et al., 2002). A small and unusual

subgroup of species of the *Cyanidium* genera has also been reported living inside caves (Ciniglia et al., 2004; Friedmann, 1964). In a previous and excellent review, Hoffmann (1994) summarizes the state of knowledge of what was known of cave *Cyanidium*.

These aerophytic epilithic "cave *Cyanidium*" would be the mesophilic members of the clade. Thus, current phylogenetic analyses support the existence of four distinct Cyanidiales lineages: the *Galdieria* spp. lineage, the *Cyanidium caldarium* lineage, the *Cyanidioschyzon merolae* plus *Galdieria maxima* lineage, and the novel monophyletic lineage of mesophilic cave *Cyanidium* spp. (Ciniglia et al., 2004). Cave *Cyanidium* often form monospecific biofilms (of photosynthetic species), which is also remarkable (Hoffmann, 1994). Although they have a long evolutionary history, only a few morphologically recognized species are known, both in the Cyanidiales as well as of cave *Cyanidium* (Ciniglia et al., 2004). Of this last group, only three species have been described based on their molecular data; *Cyanidium* sp. Monte Rotaro, *Cyanidium* sp. Sybil cave, and *Cyanidium* sp. Atacama (Ciniglia et al., 2004; Azúa-Bustos et al., 2009). This last species of cave *Cyanidium* was recently reported in a coastal cave of the Atacama Desert in Chile.

2. Chilean Cave *Cyanidium*

Based on morphological characteristics alone, Schwabe in 1936 first reported a species in two coastal caves in Chile which he assigned as *Cyanidium chilense* (Schwabe, 1936). These caves are located in the central coast of this 5,000-km-long country (Fig. 1). Later he also described another species, *Cyanidium caldarium* var. *Chilense* f. *rumpens* inhabiting rock cracks in the coast of Chile (Schwabe, 1944). However, it is known that under the microscope, species of red algae are difficult to identify based on morphology alone (Robba et al., 2006). In particular, species of the *Cyanidium* and *Galdieria* genera are indistinguishable (Ferris et al., 2005; Pinto, 2007).

The aforementioned Atacama Desert is located between 17° S and 27° S. latitude in northern Chile (Fig. 1). It is constrained on the east by the Andes Mountains and on the west by the Coastal Range.

The Atacama is the driest and probably the oldest extant desert on Earth, having experienced hyperaridity probably for the last 15 million years (Houston and Hartley, 2003; Hartley et al., 2005). To survive in these hyperarid conditions, microalgae and cyanobacteria have adapted to very low air humidity levels, an almost complete absence of rain events, highly saline soils, and high solar radiation (Bao and Gu, 2004; Dose et al., 2001; McKay et al., 2003). These harsh environmental factors may explain why parts of the Atacama Desert are almost devoid of microbial life (Dose et al., 2001; Glavin et al., 2004; Navarro-González et al., 2003). Interestingly, the Atacama Desert has been established as a model for astrobiological studies, and many research teams have conducted research on a diversity of topics related to the biology of extremophiles (Skelley et al., 2005; Warren-Rhodes et al., 2006).

Figure 1. Geographical locations of Chilean cave *Cyanidium*. A indicates the cave described by Azúa-Bustos et al. (2009). B and C, indicate the caves described by Schwabe (1936).

3. Cave Description

The Atacama cave is located in the northern coastal range of this Desert, about 20 km north of the city of Antofagasta. It is placed at the bottom of a cliff, facing south towards the Pacific Ocean (Fig. 2). The cave is approximately 35 m deep and 6 m wide, with an average height of 3–4 m, having two entrances. The sea enters the cave through one of the entrances, covering no more than one third of the ground during high tides.

Because of this, the cave has high relative air humidity (from 60% to 90%). The temperature of the walls inside the cave is cool, fluctuating around 14°C during most part of the day. Water droplets collected from the walls where the *Cyanidium* biofilm is located have a pH of 4.5. These water droplets appear to be periodic,

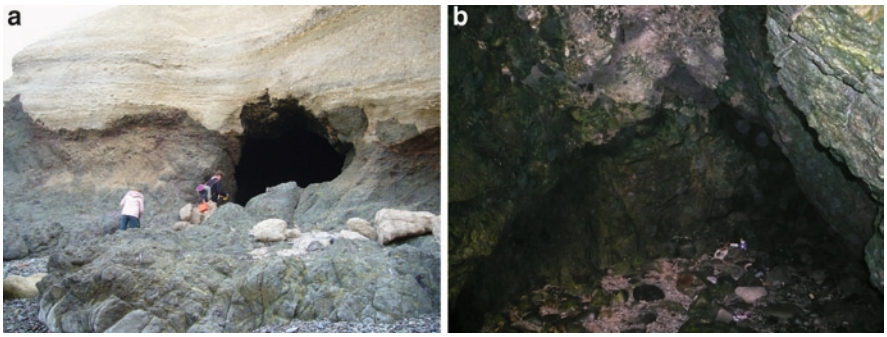

Figure 2. Atacama cave. (**a**) East entrance. (**b**) View towards the bottom of the cave.

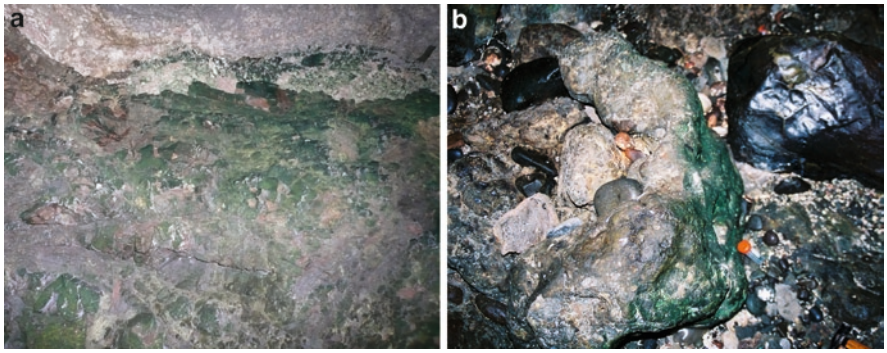

Figure 3. Cave interior. (**a**) *Cyanidium* biofilms on the left wall at the bottom of the cave. (**b**) Biofilm on rocks at the bottom of the cave. Note the development of the biofilm only on the rock face oriented towards the light coming from the east entrance.

since they were observed during the end of summer, but not by the end of fall, when the walls of the cave were drier.

The *Cyanidium* biofilms have the typical intense green-emerald color already described for cave *Cyanidium*. The biofilms are dispersed throughout the cave, always away from direct light coming from the entrances. The zone showing most biofilm development is located on the wall and part of the ceiling of the cave about 13 m from the entrance (Fig. 3). The eastern wall shows no development at all of biofilms.

The measured Photosynthetic Photon Flux Density (PPFD) values at the area where the main *Cyanidium* biofilm develops range from 1 to 3 mol $m^{-2}s^{-1}$, that is, 0.06–0.17% of the outside incident light. The bottom of the farthermost zone of the cave, where a thin biofilm can still be found, had values of 1 mol $m^{-2}s^{-1}$ (Fig. 4). The eastern wall received no light as measured by the used PPFD sensors. Thus, the areas of the cave where the observed biofilm develops are subject to extremely low light intensities, close to the measuring sensitivity of the PPFD equipment.

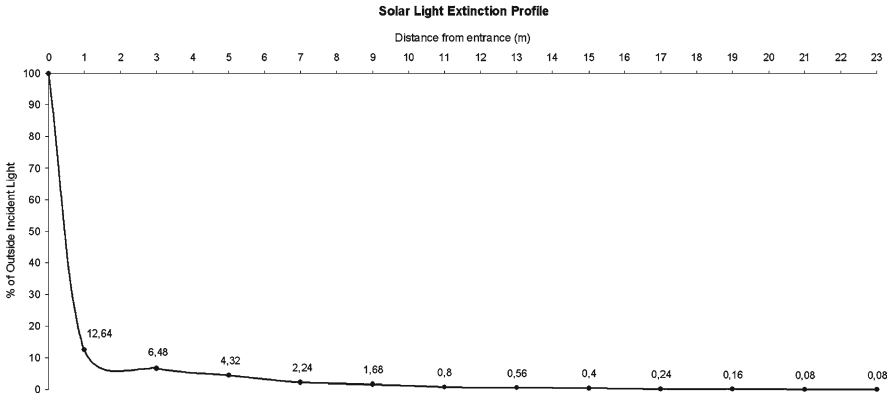

Figure 4. Light availability profile inside the cave.

It is interesting to note that the habitat description made by Schwabe in the coastal caves of central Chile is identical to the one found in the Atacama cave, which is about 1,300 km away: "the best development was found several meters from the cave entrance, at the walls and ceilings. However, areas exposed to the light (cave entrance) show no growth" (Schwabe, 1936).

This may be understood if the relatively low temperatures of this cave and the high light conditions (both visible and UV) of the areas close to the entrances are taken into consideration. It is well known that the concurrence of these two conditions determine an important stress upon the photosynthetic machinery, which cause the production of highly deleterious reactive oxygen species and photoinhibition (Murata et al., 2007). Without water limitations, cave *Cyanidium* was found as the only photosynthetic species in the biofilm growing at the end of the photic zone. The light levels at this zone may be preventing the sustained growth of other less "efficient" phototrophs. Thus, at the bottom of the cave only *Cyanidium* biofilms can be found due to their highly sensitive photosynthetic machinery, machinery that in turn may not be able to cope with the higher levels of light found closer to the entrance.

4. Atacama Cave Cyanidium Molecular Characterization

The examination of the biofilm samples scrapped from the cave walls show a homogeneous and seemingly monospecific population of *Cyanidium* cells. The spherical cells are 3–6 m in diameter, and some autospores containing cells are seen as well. The cell's single chloroplast, which occupies an important part of the cell interior, emits an intense chlorophyll autofluorescence red signal under the confocal microscope (Fig. 5). The presence of functional chlorophyll suggests that most cells are photosynthetically active, but heterotrophy can not be ruled out at this stage.

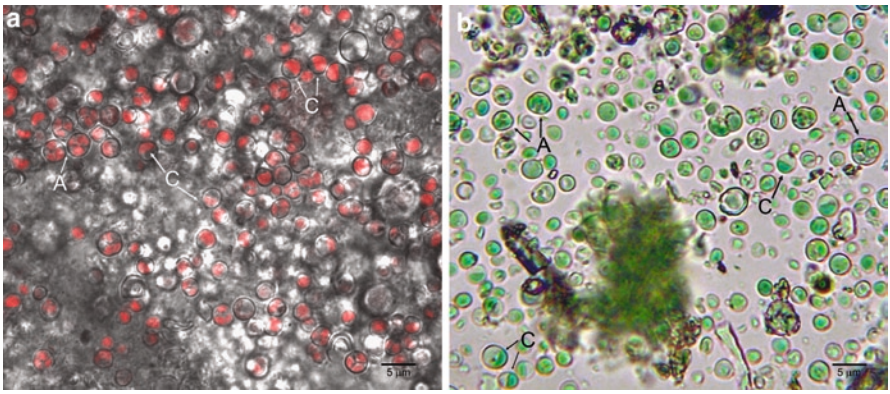

Figure 5. Micrographs of *Cyanidium* sp. Atacama cells found in biofilms of the Atacama cave. (**a**) Merged Confocal Laser Scanning Microscopy micrograph of aqueous suspension of *Cyanidium* cells extracted from the cave biofilm. The differential interference contrast (DIC) image was merged with the red fluorescence (excitation/emission 543 nm/long pass filter <570 nm) due to the autofluorescence emitted by the cell chloroplast containing chlorophyll. (**b**) Bright field micrograph of *Cyanidium* cells composed of single photosynthetic cells and small fragmented colonies. Scale bar = 5 µm. The arrows indicate chloroplasts (C) and autospores (A) containing cells.

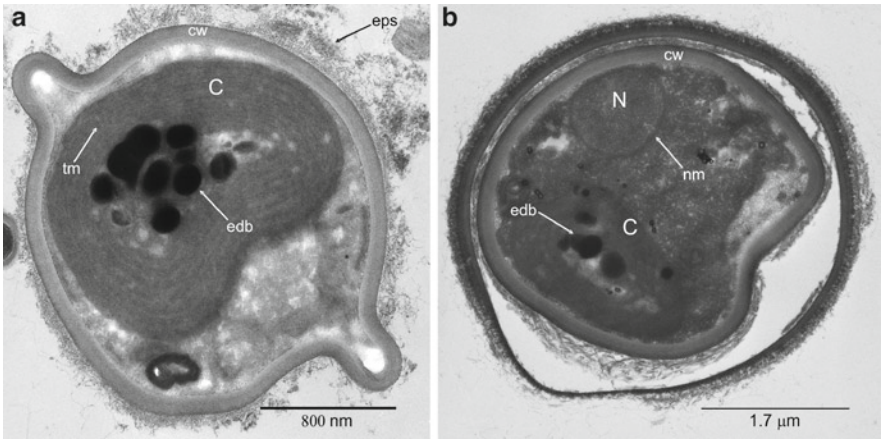

Figure 6. TEM micrograph of an ultrathin section of *Cyanidium* cells. Note the characteristic organelles and the multilayered envelopes surrounding the cells. (**a**) Scale bar = 1.4 µm. (**b**) Scale bar = 1.7 µm. C: chloroplast, V: vacuole, N: nucleus, M: mitochondria, cw: cell wall, nm: nuclear membrane, eps: exo-polysaccharide layer, edb: electron-dense bodies.

Transmission electron microscopy (TEM) show the typical ultrastructural elements previously described for these unicellular red algae. The concentrically arranged thylakoid membranes show numerous embedded phycobillisomes. Several electron-dense bodies are also observed, which in some cells appear to form two different chains at the opposite poles (Fig. 6). Scanning electron microscopy (SEM)

show the *Cyanidium* biofilm attached to the rock. Two different modes of aggregation are observed: one in which the cells are loosely associated with each other, with little or no presence of extracellular materials, and another in which the cells are embedded in a well-developed extracellular matrix (Fig. 7). In desert environments, subaerial biofilms species often secrete exo-polysaccharides (EPS) that facilitate the retention of the scarce water available for longer periods (Gorbushina, 2007). In the case of the cells aggregating without EPS, the down-regulation of EPS production at high cell densities could allow cells to redirect energy from EPS production into growth and cell division prior to a dispersal event (Nadell et al., 2008). Also, it could be a manifestation of the initial stages of the *Cyanidium* biofilm formation.

Using *Cyanidium* specific oligonucleotide primers, the 16S rRNA, *rbcL* (ribulose-l,5-bisphosphate carboxylase/oxygenase, RuBisCO) and *psbA* (photosystem II reaction center protein D1) genes of the Atacama cave *Cyanidium* were amplified. As seen in Table 1, the highest sequence that matches for these three genes correspond to the two other known cave *Cyanidium*; *Cyanidium* sp. Monte Rotaro and *Cyanidium* sp. Sybil cave. A single maximum likelihood tree for the 16S rRNA gene shows that the *Cyanidium* cave species are recovered as a monophyletic group that includes *Cyanidium* sp. Atacama and *Cyanidium* sp. Monte Rotaro as sister species with 100% of bootstrap support. *Cyanidium* sp. Sybil cave was basal to this relationship with a bootstrap support of 91% (Fig. 8).

A similar trend was observed for the single maximum likelihood tree obtained for the *psbA* gene (Fig. 9). Although a lower identity percentage is observed in the case of the *rbcL*, its closest reported relative is *Cyanidium* sp. Monte Rotaro (Table 1). The maximum likelihood tree obtained for the *rbcL* gene did not provide sufficient resolution, as Long Branch attraction persisted for the *rbcL* gene of *Cyanidium* sp. Atacama. It has been previously reported that in the Cyanidiales, ribosomal sequences are more conserved than that of the *rbcL* gene sequences (Ciniglia et al., 2004; Toplin et al., 2008; Vogl et al., 2003). *rbcL* gene sequences are

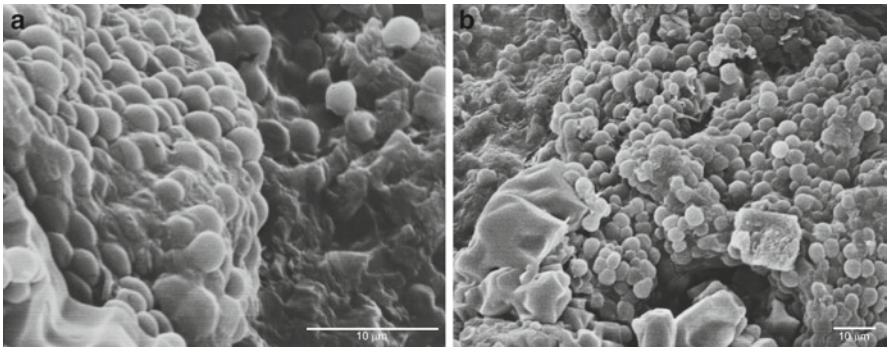

Figure 7. SEM micrographs of Atacama cave *Cyanidium.* (**a**) Biofilm conformation where the cells are well embedded in matrix of exo-polysaccharides covering the parental rock. Scale bar = 10 µm. (**b**) Biofilm where individual cells are forming loose well defined aggregates. Scale bar in meters.

Table 1. Identity percentage of selected genes of *Cyanidium* sp. Atacama with known cave *Cyanidium*.

	% of identity with *Cyanidium* sp. Atacama	
Gene	***Cyanidium* sp. Monte Rotaro**	***Cyanidium* sp. Sybil Cave**
16S rRNA	97	93
rbcL	89	79
psbA	91	86

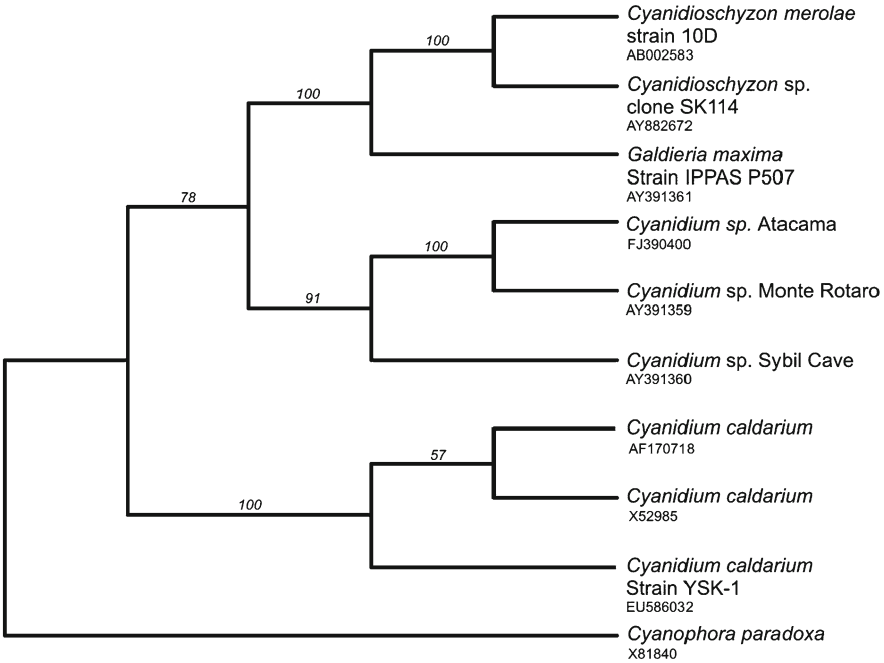

Figure 8. Maximum-likelihood tree obtained from the aligned sequences of the 16S rRNA chloroplast gene for selected Cyanidiales species. Numbers above the nodes represent 10,000 replicates bootstrap values.

known to be subjected to higher rates of sequence evolution, leading to saturation events precluding sufficient resolution in single-gene and even combined genes approaches (Toplin et al., 2008; Vogl et al., 2003). This discrepancy may be explained by the lateral transfer of the *rbcL* gene (Toplin et al., 2008). Also, previous analyses suggest that there is a high level of sequence divergence among Cyanidiales species based on environmental conditions.

Work is now in progress in trying to find the two caves originally described by Schwabe (1936). This will allow the determination of the identity of the species of

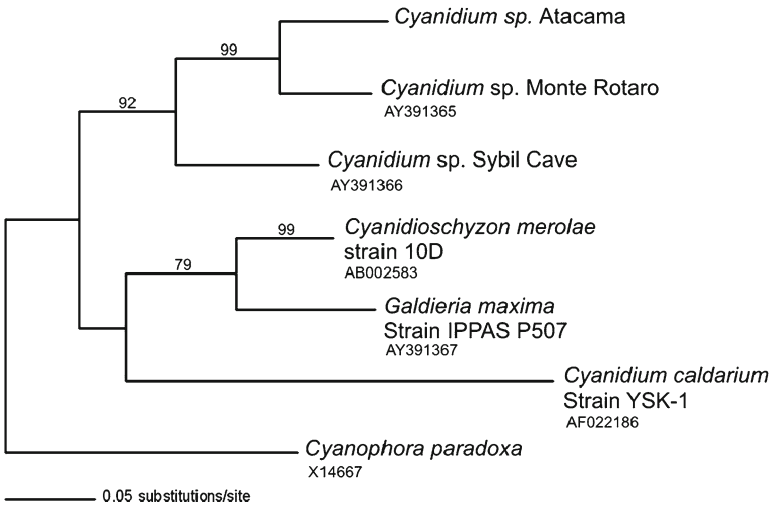

Figure 9. Maximum-likelihood tree obtained with the aligned *psbA* chloroplast gene sequences for selected Cyanidiales species. Values above the nodes represent 10,000 replicates bootstrap values.

cave algae growing in those locations, permitting future phylogenetic comparisons with the Atacama cave *Cyanidium*.

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PART 5:
BIOCHEMISTRY AND PHYSIOLOGY

Eggert
Karsten
Weinberger
Potin

Biodata of **Anja Eggert** and **Ulf Karsten**, authors of ***"Low Molecular Weight Carbohydrates in Red Algae – An Ecophysiological and Biochemical Perspective"***

Dr. Anja Eggert is since 2003 Scientific Assistant at the Chair of Applied Ecology, Institute of Biological Sciences, University of Rostock, Germany. She obtained her Diploma in 1997 from the University of Bremen and her Ph.D. in 2002 from the University of Groningen, both theses were related to the ecophysiology of polar and tropical macroalgae. Anja Eggert extended her interests to the fields of biochemistry and molecular biology of algae in various projects at the University of Rostock. Her main research focus is related to the adaptation and acclimation potential of marine red algae and other algae to environmental parameters such as temperature, salinity, and UV-stress. Anja Eggert works now in ecosystem modelling at the Leibniz Institute for Baltic Sea Research Warnemünde, Germany.

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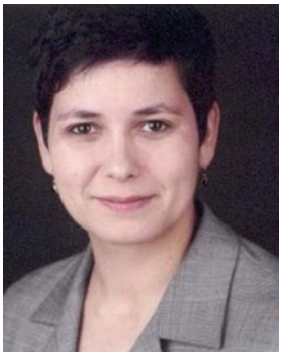

Anja Eggert

Ulf Karsten

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LOW MOLECULAR WEIGHT CARBOHYDRATES IN RED ALGAE – AN ECOPHYSIOLOGICAL AND BIOCHEMICAL PERSPECTIVE

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1. Introduction

The red algae (Rhodophyta) represent a distinct eukaryotic lineage characterized by the accessory photosynthetic pigments phycoerythrin, phycocyanin, and allophycocyanins arranged in phycobilisomes, and the absence of flagella and centrioles (Woelkerling, 1990). While some rhodophytes are unicellular, most species grow as filaments or membranous sheets of cells. The evolutionary relationships of simpler red algae, both unicellular and multicellular, have been the subject of extensive investigations for many years (Seckbach, 1994). The paleontological specimen *Bangiomorpha pubescens* from the 1,200-million-year-old Hunting Formation in the Canadian Arctic is morphologically very similar to the contemporary genus *Bangia* (Butterfield et al., 1990), and hence represents the earliest putative record for taxonomically resolvable complex multicellularity among eukaryotes, as well as for the early evolutionary origin of the multicellular red algae.

The Rhodophyta clearly constitute one of the major radiations of eukaryotes. Measured by divergence of SSU rDNA sequences within the most conservative regions, red algae are more divergent among themselves than are green algae and green plants together (Ragan et al., 1994). Although previous phylogenetic studies of the Rhodophyta have provided a framework for understanding red algal phylogeny, there still exists the need for more analysis using a broad sampling of taxa and sufficient phylogenetic information to better define the major lineages (Saunders and Hommersand, 2004; Yoon et al., 2006). The most recent study used a multi-gene approach, and based on the underlying data established a robust red algal phylogeny to identify the major clades (Yoon et al., 2006). The phylogenetic tree established by these authors identified seven well-supported lineages (classes) with the Cyanidiophyceae having the earliest divergence and being distinct from the remaining lineages, that is, the Florideophyceae, which host most of the macroscopic taxa, Bangiophyceae, Compsopogonophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae. Red algae are a large assemblage of at least 6,000 species in about 670 largely marine genera (Woelkerling, 1990).

Rhodophytes predominate along the coastal and continental shelf areas of tropical to polar regions (Lüning, 1990). Among all other algal groups red algae show the widest vertical distribution occurring from the supralittoral zone with

genera such as *Bangia* and *Porphyra* down to the deepest sublittoral of record crustose coralline species at 268 m off Bahamas (Littler et al., 1985). Red algae are ecologically important as primary producers, providers of structural habitat for many other marine organisms, in the primary establishment and maintenance of coral reefs. In addition, some red algae are economically important as source of human food and colloidal compounds found in the cell wall, such as agar, agarose, and carageenan. For this reason, extensive farming and natural harvest of red algae occurs in numerous areas of the world (Lüning, 1990).

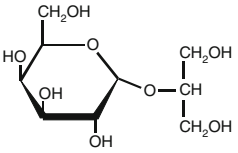
2. A High Biochemical Diversity of Low Molecular Weight Carbohydrates

During photosynthesis red algae fix inorganic carbon *via* the common plant enzyme ribulose-bisphosphate-carboxylase/oxygenase (RubisCO). However, the subsequent carbon flow into low molecular weight carbohydrates is much more diverse compared to other algal groups (Fig. 1). The main photosynthetic product in members of most orders of Rhodophyta is the heteroside floridoside (α-D-galactopyranosyl-(1-2)-glycerol). However, especially in the florideophyceaen order Ceramiales and in the more ancient lineages such as Bangiophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae (according to Yoon et al., 2006) a multitude of low molecular weight carbohydrates (LMWCs) has been identified. In addition, Rhodophytes store their energy surplus from photosynthesis in the form of floridean starch, a unique carbohydrate assembled from approximately 15 glucose units, which is, in contrast to higher plants, accumulated outside the chloroplast in the cytoplasm.

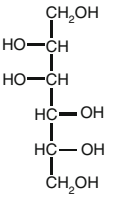
In contrast to most red algal orders, members of the Ceramiales (Florideophyceae) generally synthesize and accumulate the chemically related digeneaside instead of floridoside (α-D-mannopyranosyl-(1-2)-glycerate) (Kremer, 1978). However, it has been demonstrated that the genera *Laurencia* and *Osmundea* (Rhodomelaceae, Ceramiales) produce and accumulate floridoside and not digeneaside (Barrow et al., 1995). Also members of other red algal orders such as the Gigartinales (Florideophyceae) contain floridoside along with digeneaside (Karsten et al., 2007).

The consistent presence of digeneaside together with a new compound was noted in some members of the genus *Hypoglossum* (Delesseriaceae, Ceramiales), and a chemical survey in members of this taxon was undertaken (Karsten et al., 2005). By means of one- and two-dimensional NMR spectroscopy, the structure of this substance was proven to be digalactosylglycerol, namely 2,3-dihydroxypropyl (*α*-D-galactopyranosyl)-(1 6)-*β*-D-galactopyranoside (Fig. 1). Compared with other heterosides, this metabolite is chemically closely related to D-/L-isofloridoside known from members of the Bangiales where the glycerol moiety is also glycosidically linked via the C1-atom to the anomeric carbon of the respective galactose (Karsten et al., 1993). Surprisingly, digalactosylglycerol has never been reported for any other algal species before, even though it represents most probably the main photosynthetic product in *Hypoglossum barbatum* and *H. heterocystideum*.

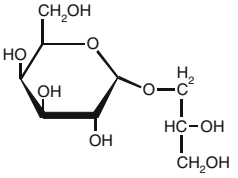
floridoside
(α-D-galactopyranosyl-1-2-glycerol)



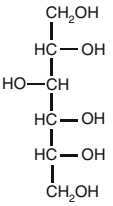
D-mannitol
(hexan-1,2,3,4,5,6-hexol)



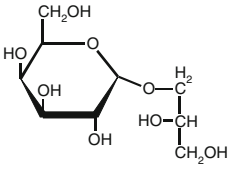
D-isofloridoside
(α-D-galactopyranosyl-1-1-glycerol)



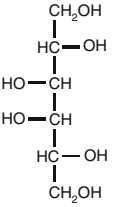
D-sorbitol
(hexan-1,2,3,4,5,6-hexol)



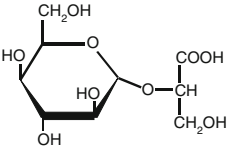
L-isofloridoside
(α-L-galactopyranosyl-1-1-glycerol)



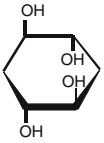
D-dulcitol
(hexan-1,2,3,4,5,6-hexol)



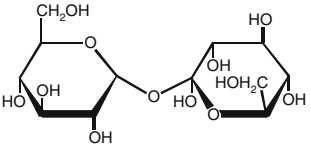
digeneaside
(α-D-mannopyranosyl-1-2-glycerate)



cyclohexan-1,2/4,5-tetrol



trehalose
(α-D-glucopyranosyl-α-D-glucopyranoside)



digalactosylglycerol
(2,3-dihydroxypropyl (α-D-galactopyranosyl)-
(1→6)-α-D-galactopyrano side)

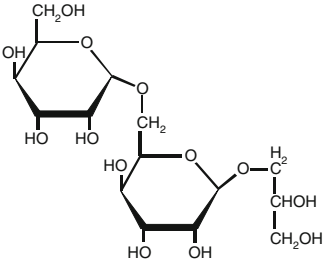


Figure 1. Typical low molecular weight carbohydrates found in red algae.

Additionally, the disaccharide trehalose was detected in several members of the Ceramiales in a more recent study on LMWCs in Florideophyceae. While some taxa of this order such as *Aglaothamnion* exhibited only trehalose, others such as *Delesseria sanguinea* showed trehalose together with digeneaside. Although the more recent data indicate that only a few Ceramialean taxa are capable of synthesizing trehalose (Karsten et al., 2007), the summarized results in the review of Craigie (1974) point to other Florideophycean species also forming this disaccharide. Therefore, this compound may be more widely distributed among the Rhodophyta than currently thought.

Also within the ancestral red algal taxa, the main photosynthetic products show a very high biochemical diversity. Most interesting is the fact that all known heterosides and polyols found in the Ceramiales are also evident in the morphologically simple, early diverging red algal lineages such as the Cyanidiophyceae, Bangiophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae (according to Yoon et al., 2006) (Eggert et al., 2007; Karsten et al., 1999, 2003).

The algae classified within the Cyanidiophyceae and Porphyridiophyceae only accumulate floridoside, that is, the heteroside most commonly found within the Florideophyceae. Furthermore, the cyclitol 1,4/2,5 cyclohexanetetrol occurs in relatively high concentrations in the unicellular *Porphyridium* sp. (Porphyridiophyceae) as the quantitatively second most important LMWC after floridoside (Craigie, 1974).

Already Lindberg (1955) provided evidence that, in addition to floridoside, members of the Bangiales (Bangiophyceae) contain an isomeric form of floridoside, isofloridoside (α-D-galactopyranosyl-(1-1)-glycerol). Wickberg (1958) later reported isofloridoside in *Porphyra umbilicalis* as an uniform mixture of D- and L-forms, but Peat and Rees (1961) found only floridoside and D-isofloridoside in this species. More recently the chemical structures and configurations of all heterosides from *Porphyra perforata* were investigated using both NMR (nuclear magnetic resonance spectroscopy) and GC-MS (gas chromatograpy-mass spectroscopy), and the occurrence of floridoside along with both D- and L-isofloridoside was verified (Meng et al., 1987). More interesting is the observation of different heteroside patterns in *Porphyra* species from different biogeographic regions in Europe, Africa, North America, Asia, and Australia (Karsten, 1999). The composition of the three compounds varied among the species studied. In *Porphyra columbina* from Australia, L-isofloridoside was always quantitatively dominant, while floridoside was the major component in *P. dioica* from the North Sea. D-isofloridoside was usually present in small concentrations, except in *Porphyra perforata* from the Pacific coast of the USA where it occurred in equal concentrations along with floridoside and L-isofloridoside (Karsten, 1999). The results point to species-specific different enzymatic activities of the underlying anabolic pathways.

Most investigated species of the Compsopogonophyceae synthesize next to floridoside and digeneaside (Karsten et al., 1999, 2003). The combination of digeneaside and sorbitol is present within the Stylonematophyceae (Karsten et al., 1999, 2003;

Eggert et al., 2007). As the Ceramialean taxa of *Caloglossa*, species of the Rhodellophyceae were also shown to contain mannitol as the only LMWC (Karsten et al., 1999, 2003; Eggert et al., 2007). Although this polyol is the main photosynthetic product of brown algae (Reed et al., 1985), records of its occurrence in the red algae remain still uncommon.

3. Low Molecular Weight Carbohydrates as Chemotaxonomic Marker

In algal taxonomy the occurrence or lack of specific carbohydrate components such as major storage compounds, cell-wall constituents, or low molecular weight photosynthates has been considered useful for distinguishing groups at several levels (Percival, 1979; Kremer, 1980; Barrow et al., 1995). Although the physiological state of an algal species and the environmental conditions are recognized to strongly influence the intracellular concentrations of low molecular weight carbohydrates (Karsten et al., 1993, 2005), the principal biochemical capability to produce particular compounds could still be useful for chemotaxonomic considerations.

Kremer and Vogl (1975) regarded the occurrence of the heteroside digeneaside within the Ceramiales as chemotaxonomically useful. More recent studies, however, indicate a more complex picture on the diversity and distribution of LMWCs among the Ceramiales. Taxa such as that with mangroves-associated genera *Bostrychia*, *Stictosiphonia*, and *Caloglossa* accumulate the sugar alcohols sorbitol, dulcitol, and mannitol (Karsten et al., 1992a, b). Furthermore, digalactosylglycerol has recently been reported as a new physiologically important LMWC in *Hypoglossum*. As most orders of the Florideophyceae typically synthesize and accumulate as main photosynthetic and reserve product floridoside and the combination of LMWCs within the Ceramiales are manifold, LMWCs have only a limited or even no chemotaxonomic value within the Florideophyceae (Karsten et al., 2007).

In contrast, the LMWC patterns within the morphologically simple, early diverging red algal lineages such as the Bangiophyceae, Porphyridiophyceae, Rhodellophyceae and Stylonematophyceae (according to Yoon et al., 2006) are more apparent. Each of these red algal classes exhibits a distinct biochemical profile and the role of LMWCs as a chemotaxonomic marker in ancestral red algae is evident.

4. Biosynthesis of Low Molecular Weight Carbohydrates

Classical radiocarbon tracer studies indicated that exogenous inorganic ^{14}C is rapidly taken up and assimilated into floridoside, which is thus acting as a major photosynthetic product (Kremer and Kirst, 1981). Although the floridoside biosynthesis was further studied and verified by Kremer and Kirst (1981) using various Florideophyceaen taxa such as *Catenella repens*, *Corallina officinalis* and *Lomentaria umbellata* as experimental systems, the anabolic pathways

for D- and L-isofloridoside in the Bangiales are still unknown. The freshwater Chrysophyte *Poterioochromonas malhamensis* (as *Ochromonas malhamensis*) is the only known algal taxon besides some bangiophyceaen taxa capable of producing L-isofloridoside (Kauss, 1977). The biosynthesis of floridoside in the Rhodophyta and of L-isofloridoside in *P. malhamensis* is initiated by a condensation reaction of L-glycerol-3-P and UDP-galactose resulting in floridoside-P and L-isofloridoside-P, respectively. These reactions are mediated by respective heteroside-P synthases (Kauss, 1977; Kremer and Kirst, 1981). Floridoside-P and L-isofloridoside-P are subsequently de-phosphorylated by specific phosphatases. In both anabolic pathways L-glycerol-3-P serves as precursor. In the case of floridoside the condensation reaction takes place at the C-2 position and in the case of L-isofloridoside at the C-1 position of glycerol. By analogy with these pathways, the precursor for the still unstudied D-isofloridoside biosynthesis is most probably D-glycerol, though this compound is a very unusual and rare metabolite.

The biosynthesis of digeneaside in red algae is initiated by a condensation reaction of L-glycerate-P and UDP-mannose resulting in digeneaside-P. This reaction is mediated by digeneaside-P synthase, and followed by a specific digeneaside phosphatase which de-phosphorylates digeneaside-P.

The biosynthesis of mannitol was studied for the first time in a red algal species, *Caloglossa leprieurii* (Florideophyceae), which is known as a typical epiphyte on mangrove aerial roots (Karsten et al., 1997). These authors verified that four enzymes regulate the size of the mannitol pool. Mannitol-1-P dehydrogenase (Mt1PDH; EC 1.1.1.17) reduces fructose-6-P to mannitol-1-P. Mannitol-1-phosphatase (Mt1Pase; EC 3.1.3.22) subsequently dephosphorylates mannitol-1-P and releases mannitol. The decomposition of mannitol to fructose is carried out by mannitol dehydrogenase (MtDH; EC 1.1.1.67) and further to fructose-6-P by hexokinase (HK; EC 2.7.1.1.). Both the anabolic and catabolic pathways involved in mannitol metabolism are known as the so-called mannitol cycle (Fig. 2).

In accordance with studies on fungi (Hult and Gatenbeck, 1979) and parasitic protozoans (Schmatz, 1989), Karsten et al. (1997) proposed that, as the reaction catalyzed by Mt1Pase is irreversible (i.e. activity of a mannitol kinase converting mannitol to mannitol-1-P could not be detected), the anabolic pathway was unidirectional. Thus, this metabolic pathway differs from that in higher plants, where mannitol is synthesized *via* mannose-6-phosphate isomerase and mannose-6-phosphate reductase (Rumpho et al., 1983). More recently, Iwamoto et al. (2003) reported the biochemical and kinetic properties of purified Mt1PDH from *Caloglossa continua*. These authors showed that this enzyme is directly regulated by salinity and consider it as the key enzyme for the regulation of mannitol biosynthesis.

The mannitol cycle has also been verified for the first time in the unicellular red alga *Dixoniella grisea* (Rhodellophyceae), which is considered as rather primitive taxa (Eggert et al., 2007). All four enzymes involved in the cycle were detected and characterized in crude algal extracts. These data provide evidence that the mannitol cycle is of ancient origin in the red algal lineage.

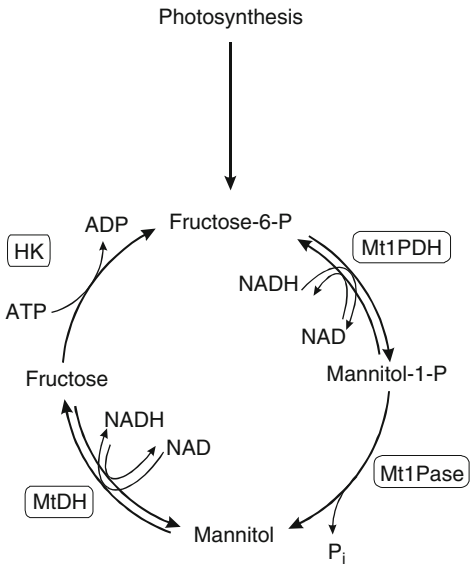


Figure 2. The mannitol cycle has been verified in *Caloglossa leprieurii* (Florideophyceae, Karsten et al., 1997) and *Dixoniella grisea* (Rhodellophyceae, Eggert et al., 2007).

In contrast to the mannitol metabolism the biosynthesis of sorbitol and dulcitol, as well as of trehalose in the Rhodophyta is completely unknown. Nevertheless, a trehalose-6-phosphate synthase gene was recently screened out from a large-DNA-fragment library constructed from *Porphyra yezoensis* (Dai et al., 2004), indicating the genotypic presence of a trehalose biosynthesis key enzyme in a representative taxa of the Bangiophyceae. This example strongly supports the usefulness of various recent genomic projects on different red algal taxa, which will give a deeper look into the molecular mechanisms of biosynthesis and regulation of low molecular weight carbohydrates.

5. Function of Low Molecular Weight Carbohydrates

Osmotic acclimation in response to salinity changes is a fundamental mechanism of salinity tolerance that conserves the stability of the intracellular milieu (homeostasis), and is essential for maintaining an efficient functional state in the cells (Kirst, 1990).

Since protein and organelle function (e.g., ribosomes, mitochondria), enzyme activity, membrane integrity, and structural macromolecules in red algae are adversely affected by increased electrolyte concentration (Kirst, 1990),

it is now generally accepted that the biosynthesis and accumulation of organic osmolytes in the cytoplasm permits the generation of low water potentials without incurring metabolic damage (Yancey, 2005 and references therein). For these organic compounds that are tolerated at high intracellular concentrations, the term 'compatible solute' was introduced by Brown and Simpson (1972). In general, the intracellular concentrations of these organic osmolytes are actively adjusted and directly proportional to external salinity (Karsten et al., 1996, Fig. 3). With exception of digeneaside, that plays not more than a minor role in osmotic acclimation, most LMWCs were reported to accumulate by a factor of approximately 1.5 between full and double strength seawater. While mannitol in *Caloglossa stipitata* even increased 3.3-fold between 33 and 60 psu, floridoside accumulated in *Bangia atropurpurea* 7.5 times between freshwater and full strength seawater. Although differing in their chemical structure, compatible solutes in red algae have some features in common: they are highly soluble (except dulcitol), in most cases have no net charges at physiological pH (except digeneaside), and are non-inhibitory at high concentrations (Kirst, 1990; Karsten et al., 1996). The interactions of these compounds with intracellular macromolecules are not completely understood and several mechanisms have been suggested. Bisson and Kirst (1995) discussed the different models to explain protection of enzyme systems: (I) binding of the solute to the protein, (II) colligative action of the solute, (III) buffering of potentially damaging changes in solution properties,

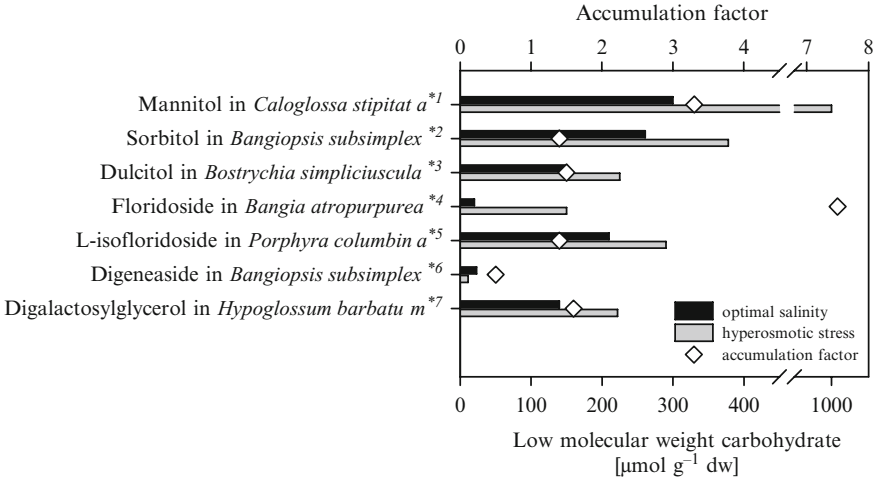


Figure 3. Accumulation of a number of low molecular weight carbohydrates under hyperosmotic stress, i.e. their function as osmotic effectors. *1: Karsten and West (1993), *2: Eggert et al. (2007), *3: Karsten et al. (1994), *4: Reed (1985), *5: Karsten et al. (1993), *6: Eggert et al. (2007), *7: Karsten et al. (2005).

(IV) inhibition of conformational changes resulting in formation of inter- or intramolecular disulfide bridges, (V) preferential exclusion of the solute from the protein surface. These models can be basically summarized into two types: (1) those that hypothesize direct solute–protein interactions and (2) those that postulate that protein stability is mediated by solute-induced changes in water structure (Roberts, 2005; Yancey, 2005). However, there is little experimental evidence in red algae for any of these models.

In freshwater cyanobacteria trehalose contributes to the osmotic acclimation (Reed et al., 1984), but plays only, if at all, a minor role as a compatible solute (Warr et al., 1988) because the three-dimensional structure of this disaccharide is generally known to interfere with enzyme function (Hinton et al., 1969). More recent publications indicate that trehalose is strongly involved in anhydrobiosis (Yancey, 2005 and references therein). During desiccation this disaccharide may bind to macromolecules and membranes by replacing water and maintaining their basic structure. Trehalose forms a glass-like state under dry conditions which contributes to the preservation of cellular structures (Yancey, 2005). In addition, freezing and heat tolerance in many organisms such as yeast, higher plants, insects, etc. is related to the presence of trehalose (Yancey, 2005). Similar physiological functions, however, have still to be experimentally proven for red algae.

Mannitol also functions as a strong antioxidant in higher plants owing to its ability to scavenge free oxygen radicals (Jennings et al., 1998). Because of the high mannitol concentrations of up to 30% of dry weight under hypersaline conditions in various *Caloglossa* species (Karsten and West, 1993) it is reasonable to assume such an antioxidative role in red algae as well. Whether the other low molecular weight carbohydrates exert a similar function has to be explored in future experiments.

Sugar alcohols in general exert multiple functions in metabolism, that is, besides their roles as organic osmolytes and compatible solutes, they can also act as antioxidants, heat protectants (stabilization of proteins), and rapidly available respiratory substrates (energy supply for a maintenance metabolism under stress and for repair processes). Consequently, particularly red algae from extreme habitats such as mangroves exhibit the capability to synthesize and accumulate these for Rhodophyta rather uncommon compounds.

Although the accumulated low molecular weight carbohydrates represent the main photosynthetic products and exert multiple physiological functions under environmental stress, some compounds play additional important roles in biotic interactions. The red alga *Delisea pulchra* (Florideophyceae) forms a water-soluble complex of floridoside and isethionic acid which acts as a chemical cue for settlement of planktonic larva of the echinoid *Holopneustes purpurascens* followed by metamorphosis into a benthic stage (Williamson et al., 2000). These authors stated that algal sugars or sugar derivatives would seem to generally meet at least two important criteria for an effective water-soluble cue: I. The potential to reach high concentrations *in situ*, and II. Some degree of source specificity.

6. Conclusion

The biochemical diversity of LMWCs in red algae is much higher as known from phycology textbooks. Although the physiological function of these organic compounds has been intensively studied in this algal group, the underlying anabolic and catabolic pathways are only partly understood. With new developments in genomics, proteomics, metabolimics, and analytical chemistry, new types of LMWCs will continue to be discovered and their biosynthetic and regulatory mechanisms elucidated.

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RED ALGAL DEFENSES IN THE GENOMICS AGE

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1. Introduction

Marine red algae are important organisms from both an ecological and an economical point of view. In most subtidal or intertidal habitats, their extremely high diversity contributes to the functioning of the ecosystems, and in coral reef ecosystems, coralline red algae play a major role in reef building. Red algae have also provided the resources to establish a fruitful aquaculture in Far East Asia, first in Japan with the development of nori cultivation since the eighteenth century and most recently in the Philippines, Indonesia and East Africa, with the farming of carrageenophytes (*Eucheuma* and *Kappaphycus*) promoted by Maxwell Doty during the 1970s (Ask and Azanza, 2002).

During the last decades, the extension of cultivation farms and hatcheries has led to an increase in the frequency of pathologies observed on various red algal crops. There are about 13 diseases caused by oomycetes, chytrids, fungi, bacteria, or unidentified agents reported from the thallus phase of *Porphyra* (Fujita, 1990), and similar pathogens were also described to provoke symptoms in the conchocelis phase (Fujita, 1990). Craigie and Correa (1996) also described the etiology of a number of infectious diseases in cultivated *Chondrus crispus* from Canada. More recently, *Kappaphycus* farming has been threatened by the occurrence of various diseases, mainly "ice–ice,", during which parts of the plant turn white from primary or secondary attacks by bacteria and fungi (Largo et al., 1995). Another problem in *Kappaphycus* farming is heavy colonization by endo/epiphytic filamentous red algae of the *Polysiphonia* type, which results in swelling of the host tissue followed by fragmentation and rotting (Hurtado et al., 2006), and problems with epiphytism are also abundant in Gracilaria farming (Friedlander et al., 1996; Leonardi et al., 2006; Neill et al., 2006). In natural populations also, dramatic epidemics such as the coralline lethal orange disease (CLOD), a severe infection affecting various coralline algae has led to the destruction of thousands of kilometers of coral reefs (Littler and Littler, 1995), and several pathologies were described at the individual level in natural populations of red algae (Correa and Flores, 1995; Correa et al., 1997; Faugeron et al., 2000).

Yet, even though they have become common problems in algal mariculture today, host–pathogen interactions have hardly been studied beyond the phenomenology of infection. Their regulation remains largely unexplored. Most algal defensive mechanisms that have so far been identified are considered as "constitutive" in the sense of "permanent" or "unregulated." However, in most cases this is due to a lack of investigation. Detailed studies reveal variations, in most cases, in algal defense with time or with environmental factors, which indicates that regulation by environmental factors probably exists. Moreover, there is increasing evidence of enemy-aroused defense in algae.

For obvious reasons, the identification of a gene product as defense-related is facilitated when its expression or activation is enemy-regulated. Some of the biochemical bases of the innate immunity of marine red algae have been uncovered recently (Potin et al., 2002; Weinberger, 2007; Cosse et al., 2008). It is the objective of this chapter to place the current knowledge about these bases of resistance or susceptibility to disease into a genomic perspective, using the concepts which have been developed in the biochemistry and genetics of terrestrial plant–pathogen interactions. This chapter also aims to address the challenge of integrating the defenses against grazing in the same conceptual framework as disease resistance.

2. Defenses Against Fouling Organisms and Pathogens

The aquatic environment generally favors development of prokaryotic and eukaryotic microorganisms, formation of biofilms, and fouling. Algae provide a substrate for microscopic and macroscopic epi- and endobionts and clearly shape the structure and composition of microbial communities in their vicinity (Lam and Harder, 2007; Lam et al., 2007). On the other hand, they are also affected by these microorganisms. Certain bacteria have been shown to provide their host algae with growth factors, nutrients, or protection from settlement by other micro- or macrofoulers (Armstrong et al., 2001; Dobretsov and Qian, 2002; Matsuo et al., 2005; Zheng et al., 2005) and non-palatable epiphytes potentially protect more palatable hosts from grazing (Wahl and Hay, 1995; Karez et al., 2000). Nonetheless, many effects of microorganisms upon algae that are more negative than positive have so far been described and algae are able to cope with these threats under most conditions because of various defense mechanisms.

Some of the negative microbial effects upon algae are relatively unspecific. For example, most micro- and macroepibionts can obviously be competitors for light or other resources. Such competitive pressure may be posed by many different organisms most of the time. As predicted by optimal defense theory (Agrawal and Karban, 1999), the corresponding algal defense mechanisms are also typically unspecific and/or constitutive. For example, *Dilsea carnosa* sheds its epidermal layer with all attached fouling organisms from time to time (Nylund and Pavia, 2005) and *Bonnemaisonia hamifera* and *Asparagopsis armata* release halogenated

secondary metabolites that inhibit growth of a broad range of associated microorganisms (Nylund et al., 2005; Paul et al., 2006a). The physiological basis and regulation of epidermis-shedding is unknown, while the chemical defense of *B. hamifera* and *A. armata* against bacteria is considered constitutive. As a consequence, little or nothing is so far known about the molecular basis of these unspecific defense mechanisms (but see also Section 5 on halogenation).

Other red algal defenses against fouling are based upon inhibition of epibiont settlement rather than growth. For example, metabolites from *Delisea pulchra* inhibit settlement of bacteria, cyprid larvae of the barnacles *Balanus amphitrite* and *B. improvisus*, larvae of the Bryozoan *Bugula neritina* and the Polychaete *Hydroides elegans*, as well as settlement of common fouling macroalgae such as *Ulva* (de Nys et al., 1995; Maximilien et al., 1998; Steinberg et al., 1998; Dworjanyn et al., 2006). The active metabolites have been identified as halofuranones (Dworjanyn et al., 1999), which are similar in structure to acylated homoserine lactones (AHL). AHL perception is important in Gram negative bacteria for the regulation of swarming and biofilm formation. AHL also regulates the swarming behavior of *Ulva* zoospores (Joint et al., 2002; Tait et al., 2005), as well as the release of spores by the red alga *Acrochaetium* sp. (Weinberger et al., 2007b). Halofuranones specifically inhibit these regulatory systems (Manefield et al., 1999; Weinberger et al., 2007b). In bacteria, halofuranones act at the LuxR homologous receptor proteins, interfering with the binding of AHLs, which ultimately leads to degradation of the receptor protein (Manefield et al., 2002). Probably as a consequence of halofuranone excretion, the bacterial community associated with *D. pulchra* is dominated by Gram positive species, although Gram negative species do usually dominate on seaweeds (Steinberg and deNys, 2002). The bacterial communities that are associated with macroalgae often provide further settlement cues for macrofoulers, which has in particular been studied with coralline crustose algae (Wieczorek and Todd, 1998; Steinberg and deNys, 2002). Shifts in the community composition of alga-associated bacterial communities may potentially result in changed availability of such cues and subsequently in changed intensity of fouling (Steinberg and deNys, 2002).

However, so far relatively few other examples of epibiont settlement inhibition by red algal secondary metabolites have been reported, and observed effects have usually been limited to a lesser spectrum of target organisms than in *D. pulchra*, which may indicate that this type of defense is less widespread than has been expected (de Nys et al., 1996b; Walters et al., 1996; Steinberg et al., 1998; König et al., 1999; Nylund and Pavia, 2003, 2005). As with inhibitors of microbial growth, the regulation of settlement inhibitors by algae is largely unexplored. However, there is substantial quantitative variation in halofuranones in *D. pulchra*, not only at spatial, but also at temporal scales (Wright et al., 2004), suggesting that their excretion may be induced or activated.

More is known about the regulation of some model interactions of algae and pathogens. Alga–pathogen interactions may be highly specific and characterized by typical symptoms (For reviews see Fujita (1990) and Correa (1996)).

For example, various oomycetes are able to infect red algae, but the spectrum of compatible hosts is usually narrow (For a recent overview, see West et al., 2006). For example, a strain of *Olpidiopsis* sp. from Madagascar that had been isolated from *Bostrychia moritziana* was also able to infect certain isolates of *B. tenella*, *B. radicans*, and *B. radicosa*, but not others (West et al., 2006). Five other species of *Bostrychia* were ultimately resistant, as were six different species of *Stictosiphonia, Lophosiphonia, Neosiphonia*, and *Polysiphonia*. Interestingly, *Bostrychia* isolates originating from Madagascar were in most cases susceptible, and strains from other parts of the world mostly resistant, which seemingly indicates the existence of pathovars (strains of the same species with distinctive pathogenicity due to coevolution with different hosts) among algal pathogens. In the case of *Pythium porphyrae*, an oomycete that settles only on red algae, the basis of host specificity has been elucidated in part: zoospore attachment, encystment, and appressorium formation are induced by porphyran and other sulphated galactans that form the cell wall matrix of red algae (Uppalapati and Fujita, 2000a). However, *P. porphyrae* ultimately only infects *Porphyra yezoensis* and *Bangia atropurpurea* and protoplast fusion of *P. yezoensis* with the resistant *Porphyra tenuipedalis* resulted in hybrids with reduced susceptibility (Uppalapati and Fujita, 2000b). Further signals are therefore probably involved in the infection process, with increasing specificity leading to selective infection.

Associations between red algae and endophytic pathogens provide further examples of host recognition through the perception of cell wall galactans. For example, the endophytic green alga *Endophyton ramosum,* the causative agent of green patch disease in the red alga *Mazzaella laminarioides,* discriminates between agarophytes and carrageenophytes and is unable to penetrate or develop normally on agarophytic algae (Sanchez et al., 1996). Even more specific, another green algal endophytic pathogen, *Acrochaete operculata,* infects carrageenophytes containing lambda-carrageenan more successfully than carrageenophytes containing kappa-carrageenan. When zoospores of *A. operculata* settle and germinate, the vegetative filaments of the parasite completely invade sporophytic fronds of *C. crispus*, which contain lambda-carrageenan. In contrast, gametophytic fronds – containing kappa-carrageenan – are not infected beyond the epidermis and outer cortex (Correa and McLachlan, 1991). Kappa- and lambda-type carrageenans were shown to control endophyte penetration, with lambda-carrageenans increasing and kappa-carrageenans reducing the endophyte virulence (Bouarab et al., 1999).

Many of the microorganisms that are associated with virtually healthy macroalgae have the enzymatic capacity to disintegrate tissues of their host. For example, about 1% of the cultivatable bacteria associated with healthy *Gracilaria* were capable of degrading its agar cell wall matrix (Weinberger et al., 1994). Similar observations have been reported for other agarophytes and carrageenophytes (Largo et al., 1995; Jaffray et al., 1997). Degraders of phycocolloids are usually rare in nonalgal environmental samples (for this reason agar is traditionally used for the gellification of microbiological media), which indicates that their concentration on macroalgae has coevolutionary reasons. Not only symptoms

such as ice–ice, but also massive increases in absolute and relative numbers of phycocolloid degraders are usually observed on stressed algal tissues with a reduced capacity for autodefense (Weinberger et al., 1994; Largo et al., 1995), suggesting that the microorganisms involved are opportunistic pathogens.

3. Enemy Recognition

An increasing number of observations indicate that red algae are able to perceive enemies and their activity and to respond to them. This is not only indicated by ecological studies which reported induced defenses against herbivores (see Section 7), but also by molecular and physiological studies of model alga–pathogen interactions. These later studies identified some of the primary molecular signals that indicate pathogen presence or activity to red algae. For example, it has been shown that red algae related to the genus *Gracilaria* have the capacity to perceive agar oligosaccharides that are released during enzymatic attacks of opportunistic pathogens upon their cell wall matrix (Weinberger et al., 1999). This perception is highly specific for agar oligosaccharides with a free reducing end and consisting of at least four monosaccharide residues, and particularly sensitive toward oligosaccharides consisting of 16 residues or more (Weinberger et al., 2001). Further, the physiological response of *Gracilaria* sp. to agar oligosaccharides resulted in an elimination of associated microorganisms, and in particular of agar degraders (Weinberger and Friedlander, 2000a). Taken together, these findings suggest that innate receptors for these molecules must be present in *Gracilaria*, similar as in vascular plants for oligogalacturonans and in kelps for oligoguluronans. *Chondrus crispus* does not respond in an analogous way to carrageenan oligosaccharide, but to cell-free extract of its endophytic green algal pathogen *Acrochaete operculata*, which activates defense responses in the relatively resistant gametophyte (Bouarab et al., 1999). *C. crispus* also responded with an upregulation of haloperoxidase – suspected to be a defense-related protein (see Section 5) – when challenged with lipopolysaccharide from the outer envelope of Gram-negative bacteria (Weinberger, 2007). *Gracilaria* sp. responded physiologically to specific cellulose degradation products, as well as to peptides that were excreted by a *Cytophaga*-like associated bacterium (Weinberger and Friedlander, 2000b).

4. The Oxidative Burst

Many observations indicate that emission of reactive oxygen species [ROS; including superoxide ions (O2-•), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•)] mediates host–pathogen interactions in red algae, similar as in other algae, plants, and animals. Physiological generation of ROS may occur in numerous reactions in mitochondria, chloroplasts, peroxisomes, and other cellular elements and is frequently a consequence of stress (Dring, 2005). However, in biotic interactions,

emission of ROS is most often triggered by cell–cell recognition, involving perception of a molecular signal at the cell membrane (Potin, 2008).

A transient release of H_2O_2 was observed as a first physiological indication of agar oligosaccharide perception in *Gracilaria* sp. (as *G. conferta*) (Weinberger et al., 1999), followed by a refractory state of 6 h, which is typical for receptor-mediated oxidative bursts (Weinberger et al., 2005b). As could be shown by transmission electron microscopic observation of the H_2O_2-dependent formation of ceriumperoxide from cerium chloride, the site of ROS production was the plasma membrane of epidermal and subepidermal cells (Weinberger et al., 2005b). Further analysis of the oxidative burst response revealed that it was highly sensitive to diphenylene-iodonium (DPI), an inhibitor of NADPH-dependent enzymes, as well as to inhibitors of flavoenzymes. The authors therefore concluded that plasma membrane-located NADPH oxidase (which is a flavo-enzyme) was probably the source of ROS after elicitation of *Gracilaria* sp (Weinberger et al., 2005b), similarly as after elicitation of oxidative bursts in vascular plants and metazoans.

The interaction between *C. crispus* and its green algal endophyte *A. operculata* provides another example of the involvement of a NADPH oxidase homologue into red alga–pathogen interactions. Gametophytes of *C. crispus*, when challenged with cell-free extracts of *A. operculata*, generated an oxidative burst that was sensitive to DPI. Moreover, when incubated with DPI the gametophytes lost their resistance to *A. operculata* infection (Bouarab et al., 1999). These results therefore demonstrate that the oxidative burst is an essential element in *Chondrus* immunity. The identity of NADPH oxidase in *C. crispus* was recently elucidated. A single copy gene encoding a homologue of respiratory burst oxidase gp91^{phox}, named *Ccrboh* has been identified from the red alga *C. crispus* (Hervé et al., 2006). A search performed using the *Ccrboh* gene in available algal genome and expressed sequence tag (EST) databases identified sequences showing common features of NADPH oxidases in the red, unicellular *Cyanidioschyzon merolae* and *Porphyra yezoensis*, as well as in other algae such as diatoms (Hervé et al., 2006). Phylogenetic analysis revealed that NADPH oxidase homologs in red algae and diatoms are relatively closely related and emerged early in evolution from a common ancestor of the ferric reductase and NADPH oxidases (Hervé et al., 2006). Interestingly, in comparison with control thalli, expression of *Ccrboh* is induced and maintained at high levels for at least 24 h following the inoculation of *C. crispus* gametophytes with zoospores of *A. operculata*. Induction of *Ccrboh* mRNA accumulation occurred when germinating zoospores attempt to penetrate through the host cell wall, confirming the involvement of a DPI-sensitive NADPH oxidase in the defensive oxidative burst response (Bouarab et al., 1999).

However, NADPH oxidase is not the only ROS-generating enzyme that may be activated during red algal defense responses. For example, subcellular examination of *G. chilensis* after challenge with agar oligosaccharide showed that H_2O_2 release after exposure to agar oligosaccharides was located in the outer cell wall and not at the plasma membrane (Weinberger et al., 2005b). The response was sensitive to inhibitors of metalloenzymes and flavoenzymes, but not to DPI, and no refractory state was

observed, confirming that NADPH oxidase was not involved and that the oxidative burst activation was not linked to a receptor. Instead, the release of H_2O_2 was correlated with accumulation of an aldehyde in the algal medium, suggesting that an oxidase is present in the apoplast of *G. chilensis* that directly acts on agar oligosaccharide. The enzyme acted on all agar oligosaccharides that were larger than disaccharides and was inhibited by reduced agar oligosaccharides (Weinberger et al., 2005b). Agar oligosaccharide oxidase displays a defensive value. *G. chilensis* plants with a high potential for oxidation of agar oligosaccharides proved to be less susceptible to settlement by the epiphytic red alga *Acrochaetium* sp. than plants with a low potential (Weinberger et al., 2005b).

As revealed by native polyacrylamide gel electrophoresis, several isoforms of agar oligosaccharide oxidase were expressed in *G. chilensis*. The expression was not only affected by water temperature (Weinberger et al., 2005b), but elicitation of *G. chilensis* with agar oligosaccharide triggered a particularly strong expression of the enzyme within 24 h, which indicates that agar oligosaccharide perception took place, despite the fact that an oxidative burst was not activated (Weinberger et al., 2010). A similar increase of agar oligosaccharide oxidoreductase after challenge with agar oligosaccharide was also observed in *G.* "*conferta*," as well as in 12 out of 14 other Gracilariacean species that were tested (Weinberger et al., 2010). Most species of this family thus appear to perceive agar oligosaccharides. In contrast, a phylogenetic analysis revealed that only relatively recent clades within the family activate NADPH oxidase after the perception (Weinberger et al., 2010).

Release of H_2O_2 by an enzyme other than NADPH oxidase was also observed in the interaction of *C. crispus* with *A. operculata*. Contact of *A. operculata* with kappa-oligocarrageenans enhances secretion of the nitrogen storage compound l-asparagine (l-Asn), which in turn induces a release of H_2O_2 by *C. crispus* (Weinberger et al., 2002). This release is insensitive to DPI and correlated with release of ammonium and of a carbonyl compound into the medium. The reaction is therefore apparently catalyzed by an apoplastic L-amino acid oxidoreductase (Weinberger et al., 2005a), a view which was further supported by the identification of a homolog of l-amino acid oxidase as one of the ten most expressed proteins in *C. crispus* (Collén et al., 2006b). The H_2O_2 concentrations generated by *C. crispus* in the presence of physiologically relevant amounts of L-Asn were shown to be sufficient to significantly prevent settlement of *A. operculata* zoospores (Weinberger et al., 2005a).

5. Oxidative Burst-Associated Halogenation

Halogenated compounds, abundant in marine organisms, have been proposed to play a defensive role in marine algae because they are known to have potent antibiotic activities (Wever et al., 1991). As reviewed by Kladi et al. (2004), halogenated metabolites are very common and diverse in marine red algae and their large number

illustrates the importance of halogen metabolism in various physiological conditions. Brominated compounds dominate this chemical repertoire, even though chloride is much more abundant in seawater. In comparison with brown algae, iodinated compounds are also poorly represented (Leblanc et al., 2006). Apart of few exceptions, the biological and ecological functions of these compounds have been hardly investigated. As mentioned above (Section 2), brominated furanones of *D. pulchra* control bacterial colonization of surfaces by specifically interfering with quorum sensing-mediated gene expression at the level of the LuxR protein (Manefield et al., 2002). This may illustrate the evolution of specialized halogenation functions, in relation with signalling processes.

In red algae, a strong link was established between environmental stress, oxidative stress, and an increased production of halogenated low-molecular-weight carbon skeletons, referred to as volatile halogenated organic compounds (VHOCs) (Mtolera et al., 1996). This has led some authors to propose that halogenated compounds should only be secondary waste products of ROS detoxification processes (Pedersén et al., 1996a; Manley, 2002). VHOCs arise from the activity of vanadium-dependent HPO, which catalyze the oxidation of halides (X^-) into hypohalous acids (HXO) (Butler and Carter-Franklin, 2004). As highly electrophilic agents, hypohalous acids can react with a variety of organic compounds, probably including membrane unsaturated lipids, to generate VHOCs, that are often viewed as defense compounds.

Reminiscent of the halogenation response, which is driven by the oxidative burst in mammalian phagocytes (Gaut et al., 2001), agar oligosaccharides were shown to elicit a release of hydrogen peroxide that resulted in an immediate increase in the brominating activity of the red alga *Gracilaria* sp. (Weinberger et al., 1999). This reaction is responsible for the bleaching of thallus tips described earlier (Friedlander and Gunkel, 1992) and similar to the symptoms of the "ice–ice" white powdery disease in *Eucheuma* and *Kappaphycus* species (Lavilla-Pitogo, 1992; Largo et al., 1995; Pedersén et al., 1996b). Recent investigations have confirmed that activation of NADPH oxidase by agar oligosaccharides causes increased production of a bouquet of VHOC's in *Gracilaria* sp. (Weinberger et al., 2007a), and activation of NADPH oxidase by pathogen extracts in *C. crispus* results in the same effect (K. Bouarab, B. Cocquempot., F. Weinberger, P. Morin, B. Kloareg, P. Potin., 2003, unpublished results; Bouarab, 2000). Interestingly, after activation of agar oligosaccharide oxidases in *G. chilensis* (Weinberger et al., 2007a) or of amino acid oxidase in *C. crispus* (Bouarab et al., 2004; Weinberger et al., 2002, 2005a) the production of VHOCs remained unchanged, suggesting that membrane-associated ROS production and signaling events are required to provide additional substrates for vanadium haloperoxidases.

In red algae, VHOCs seem then to have an important physiological role in activated defense responses, acting as biocidal or repelling substances against microorganisms and herbivores, but also preventing the settlement of epiphytic or pathogenic algae. Bromoform produced by the red algae *Corallina pilulifera* and *Lithophyllum yessoense* was shown to inhibit the growth of epiphytic diatoms

(Ohsawa et al., 2001). In the red alga *Asparagopsis armata*, bromoform and dibromoacetic acid are the dominant brominated compounds among an impressive number of metabolites (Kladi et al., 2004) which are stored in specialized gland cells (Paul et al., 2006c). When released at the surface of the thallus, they display antibiotic activity against epiphytic bacteria (Paul et al., 2006a). They are also feeding deterrents of mesograzers as inferred from the increased consumption of bromide starved algal cultures (Paul et al., 2006b). In *C. crispus* gametophytes, bioassays conducted to test the toxicity of VHOCs for spores and germlings of the pathogenic endophyte *A. operculata* have shown that three compounds – ethyl iodide, bromoform, and chloroform – reduce survival rates of the infecting stages of *A.operculata* (Bouarab et al., unpublished results). The production of these compounds by *C. crispus* is enhanced upon challenge with cell-free extracts of *A. operculata* (Bouarab et al., unpublished results).

Red algae feature a variety of constitutive or inducible vHPO isoforms that have mainly iodinating or brominating activity (Butler and Carter-Franklin, 2004; Leblanc et al., 2006). Recently, bromoperoxidase activity was detected in marine macroalgae belonging to the families Gracilariceae (Weinberger et al., 2007a) and Solieriaceae, including *Solieria robusta, Eucheuma serra*, and *K. alvarezii*, and a vBPO from the former species was purified to homogeneity (Kamenarska et al., 2007). Red algal vBPOs are very well characterized at the molecular and structural levels in the genus *Corallina* (Oshiro et al., 2002; Littlechild, 2002). Four closely-related cDNAs are available for *Corallina* spp. vBPOs. The native wild-type vBPO enzyme is expressed in low yields in *C. officinalis* and recombinant expression of vBPO's in *Saccharomyces cerevisiae* or *E. coli* is predominantly in the form of insoluble inclusion bodies (Oshiro et al., 2002), thereby necessitating refolding. A successful protocol for such a refolding has been published recently (Coupe et al., 2007) and will allow further study of enzyme specificity for halide oxidation and other aspects of the controversial stereoselectivity of vBPOs (Butler and Carter-Franklin, 2004; Leblanc et al., 2006). In *C. crispus* also, a full length cDNA encoding a vBPO has been cloned and its overexpression is in progress (A. Cosse, L. Delage, P. Potin, C. Leblanc, unpublished results). The recent EST approaches in *C. crispus* demonstrated that it expresses both a vanadium-dependent BPO and an enzyme similar to hem-containing myeloperoxidase (Collén et al., 2006b).

6. Oxylipin Signals

In higher plants, lipid peroxidation (LPO) is usually associated with stress situations (Howe and Schilmiller, 2002) and often correlates with cell death (Cacas et al., 2005). Indeed, polyunsaturated fatty acids (PUFAs) are susceptible to oxidation by free radicals and thus, an increase in LPO is often considered to indicate membrane degradation or dysfunction (Davoine and Farmer, 2007). In addition to an increase in free radical-mediated LPO, enzymatic production of fatty acid hydroperoxides can be observed (Montillet et al., 2004). In red algae also, signalling cascades involving

compounds derived from the oxidative metabolism of PUFAs were shown to operate, providing the first demonstration that oxylipins have a biological role in red algal immunity and defense mechanisms (Bouarab et al., 2004). Two lipoxygenase isoforms, which were specific for the metabolism of linoleic acid, were upregulated following the oxidative burst in *C. crispus,* and lipoxygenase inhibitors abolished the natural resistance of *C. crispus* gametophytes, showing that the induced resistance which is dependent of the oxidative burst involved the downstream activation of the oxylipin pathways (Bouarab et al., 2004). The wound response of the red alga *G. chilensis* involves the release of free fatty acids as well as the hydroxylated eicosanoids, 8R-hydroxy eicosatetraenoic acid (8-HETE), and 7S,8R-dihydroxy eicosatetraenoic acid (7,8-di-HETE) (Lion et al., 2006). While the release of free arachidonic acid and subsequent formation of 8-HETE is likely controlled by phospholipase A, 7,8-di-HETE production is independent of this lipase. This dihydroxylated fatty acid might be directly released from galactolipids that contained 8-HETE or 7,8-di-HETE (Lion et al., 2005).

Although its occurrence in red algae remains controversial (Pohnert, 2004; Cosse et al., 2006), the methyl ester of the plant stress hormone jasmonic acid (MeJA) was revealed to be a powerful activator of the fatty acid oxidation in *C. crispus*. It promoted the liberation of C20 and C18 PUFAs and the corresponding oxygenated derivatives and cyclopentenones, such as prostaglandins (Bouarab et al., 2004; Gaquerel et al., 2007). Addition of MeJA to *C. crispus* also induced increased activities of enzymes that are potentially involved in defense reactions, such as shikimate dehydrogenase and phenylalanine ammonium lyase, and of an enzyme that hydroxylates polyunsaturated fatty acids (Bouarab et al., 2004; Gaquerel et al., 2007). The transcription of defense-related genes such as glutathione S-transferase (GST) and NADPH oxidase (see Section 4) was also upregulated after MeJA addition (Gaquerel et al., 2007; Hervé et al., 2006). Transcription analysis of the *C. crispus* NADPH oxidase gene *Ccrboh* revealed that it is overrexpressed after incubation in the presence of MeJA, derived from linolenic acid, and 12- hydroperoxy eicosatetraenoic acid (12-HpETE), derived from arachidonic acid (Hervé et al., 2006). The repertoire of genes generated by the EST libraries from *C. crispus* has for then been used to construct the first cDNA microarray for a macroalga and to analyze expression profiling of *C. crispus* after exposure to the plant stress hormone methyl jasmonate (Collén et al., 2006a). The study showed that 6% of the genes studied showed a response to the addition of MeJA and the most dynamic response was seen after 6 h. A comparison between different functional groups showed an upregulation of stress-related genes and a downregulation of genes involved in energy conversion and general metabolism. Interestingly, the gene of 3-deoxy-7-phosphoheptulonate synthase, which is the first enzyme of the shikimate pathway, is upregulated by MeJA. This confirms the regulation of secondary metabolite synthesis by the oxylipin pathway, which was previously discovered using biochemical approaches (Bouarab et al., 2004).

At the molecular level, only a few red algal genes involved in fatty acid metabolism have so far been characterized. A cDNA encoding a putative 12-lipoxygenase

was identified in the gametophyte of *Porphyra purpurea* (Liu and Reith, 1994) while a polyenoic fatty acid isomerase, which converts arachidonic acid into a conjugated triene, was purified and cloned from *Ptilota filicina* (Zheng et al., 2002).

In higher plants, hydroperoxy FAs produced by lipoxygenase are substrates for several enzymes that contribute also to the synthesis of antifungal compounds such as aldehydes and divinyl ethers, and a variety of plant-specific volatiles (Shah, 2005). It will be of special interest to mine genes encoding such enzymes in the growing genomic resources from red algae and to use them as probes in gene expression studies. Expression patterns may then be correlated with metabolite profiles, similar as in studies that have already been conducted with red algal oxylipins (Bouarab et al., 2004; Lion et al., 2005; Gaquerel et al., 2007) or with green algal (Akakabe et al., 2000) and brown algal (Boonprab et al., 2003) aldehydes and intermediates of FA alpha-oxidation.

7. Defenses Against Herbivores

Algae are often subject to more intense grazing than terrestrial plants (Cyr and Pace, 1993). Apart from associational defenses, which often exist (Wahl and Hay, 1995; Karez et al., 2000), algal resistance against a specific predator is in most cases based upon low palatability, which may result from structural toughness, unsuitable nutritive composition, or accumulation of chemical defense compounds (Paul et al., 2001). Numerous reports of red algal crude extracts deterring grazers exist, but the secondary metabolites responsible for low palatability are only known in few cases. In *Plocamium cartilagineum* from Antarctica halogenated monoterpenes deterred amphipods but not sea stars (Ankisetty et al., 2004). Defense against fishes by the monoterpene ochtodene was reported for *Portieria hornemanni* from Guam (Wylie and Paul, 1988) and *Ochtodes secundiramea* from Martinique (Paul et al., 1987). In *Laurencia obtusa* from Brazil the sesquiterpene elatol inhibited feeding by a crab and a sea urchin (Pereira et al., 2003). In *Delisea pulchra,* a relatively wide spectrum of grazers was deterred by halogenated furanones (Wright et al., 2004).

Information about the enzymatic and genetic basis of the biosynthesis of these compounds is still scarce. Monoterpenes and sesquiterpenes probably result from either the mevalonate-dependent or the mevalonate-independent isoprenoid pathway, which have both been detected in red algae (Pelletreau and Targett, 2008). Only low incorporation of bicarbonate, acetate, and mevalonate was observed during biosynthesis of halogenated monoterpenes by *P. cartilagineum* (Barrow and Temple, 1985), but the authors interpreted this as an indication that the isoprenoid pathway was involved.

The concentrations of feeding deterrants often vary with tissue age (de Nys et al., 1996a), individual (Puglisi and Paul, 1997; Wright et al., 2004) or other factors (Paul et al., 2001). Light, but not nutrient availability influences the presence of ochtodene in *P. hornemanni* (Puglisi and Paul, 1997). Reports of reduced palatability of red algae in response to herbivory have also recently been published.

Such induced antigrazing defense has been observed in *Pterocladia capillacea* (Weidner et al., 2004), *Hypnea pannosa* (Ceh et al., 2005) and *Galaxaura diessingiana* (Diaz et al., 2006) in response to amphipodes. Similarly, grazed *Phyllophora pseudoceranoides, Furcellaria lumbricalis*, and *Polyides rotundus* were less palatable to isopodes than ungrazed specimens (Toth, 2008; Rohde and Wahl, 2008). Such an effect was also observed when *Delesseria sanguinea* was exposed to the isopode *Idotea balthica* (Rohde and Wahl, 2008), but not when it was exposed to *Idotea granulosa* (M. Molis et al., unpublished manuscript). Toth and Pavia (2007) conducted a meta-analysis of 40 different alga-grazer combinations (including 16 red algal species) and concluded that induced defenses were less abundant in red seaweeds than in other algae. This was confirmed by Jormalainen and Honkanen (2008), who analyzed nearly the same data set. In contrast, a similar abundance of induced defenses against grazing in red seaweeds as in brown macroalgae was detected in the framework of the GAME-project (M. Molis et al., unpublished manuscript). This global experiment screened 62 alga-grazer combinations, including 20 red algal species.

The molecular basis of induced red algal defenses against herbivory is still unknown. Vascular plants often respond to wounding. Wounding results in cell disintegration, in contact of phospholipases and lipids, subsequently in release of unsaturated fatty acids, and eventually in the formation of oxylipins (Kessler and Baldwin, 2002). Similar effects of wounding are also known from red macroalgae (see Section 6) and it therefore appears as possible that wounding by enemies may induce antiherbivore defenses. However, a function of other chemical cues can currently not be excluded. Clearly, the discovery of enemy-aroused red algal defense against herbivores promises a faster future progress in the identification of antigrazing-related gene products.

8. Integrating Gene-Regulated Responses into Studies of Red Algal Defenses

The capability to monitor gene-regulated responses is essential to delineate the various steps that lead from the perception of elicitors to the establishment of efficient defenses against pathogens or herbivores. Powerful genomic approaches have now become available for the main algal models mentioned in this chapter and have allowed researchers to mine putative defense genes and identify the important metabolic pathways for the establishment of resistance. The analysis of an EST database of protoplasts from *C. crispus* gametophytes yielded a high proportion of detoxification and heat-shock proteins (Collen et al., 2006a), validating this approach for the discovery of stress-related proteins in algae. Similar approaches for the identification of genes that are related to biogenic stress appear as promising. A total of ten 154 ESTs are currently (January, 2007) available from the leafy gametophyte and 10,265 from the filamentous sporophyte of the edible red alga *Porphyra yezoensis* (Asamizu et al., 2003), 5,318 from the sporophyte of *Porphyra haitanensis* (Fan et al., 2007) as well as 8,147 from *Gracilaria changii* (Teo et al., 2007).

Similarly, with increasingly powerful methods for the identification of molecular markers, our knowledge of the genetic structure of natural populations of red algae is growing rapidly (M. Valero, 2008, personal communication). This gain in knowledge has not yet been applied in order to improve seedstock for cultivated algal species, mainly because of a lack of coupling of experienced researchers in population biology with those working on strain development. This innovative and multidisciplinary approach should bring a wealth of new fundamental results, for example, on the bases of disease resistance in marine algae and on the genetic polymorphism of red algae, as well as new solutions and genetic resources for sustaining the productivity of seaweed farms.

A sophisticated, but possibly more precise approach might be the comparative monitoring of defense-gene expression patterns, either in controlled mesocosm experiments or in natural populations experiencing different grazing or fouling intensities or infectious diseases. Using quantitative screening methods allows it to identify transcripts that are upregulated, stable, or downregulated in response to specific pests. Regulatory gene networks that control the interplay of defense pathways might be identified in this way. As mentioned above, the development and optimization of screening tools, such as dedicated DNA chips or filter arrays has been conducted in *C. crispus* (Collén et al., 2006a) and *L. digitata*. These new molecular tools are now available for the identification of major defense-related genes and for the assessment of differential gene expression patterns among populations or environments. Beneficial complementary approaches are the recently emerging application of proteome analysis in red algae (Wong et al., 2006), as well as the development of quantitative high- or medium- throughput methods for metabolite profiling of several individuals or populations of a given organism (LaBarre et al., 2004).

Knowledge of the complete genome of the florideophyte *C. crispus* by the end of 2010 (J. Collén, 2010, personal communication) should be of enormous help to identify new candidate defense genes and gene functions that are relevant for the resistance against pathogens or grazers. In addition, the Joint Genome Institute in the USA has launched an initiative for 2008 in order to sequence the genome of the bangiophyte *Porphyra purpurea*, which should increase molecular and functional approaches in this red algal model of considerable economic importance for Far East Asia. The access to these two genomes will allow for comparative genomics approaches among the two main macroalgal clades, which may help to better understand the evolution of innate immunity mechanisms and the adaptation of resistance to biotic agressors. Furthermore, the availability of pangenomic DNA microarrays will be an important tool for investigating the signaling function and pathways of defense regulators (Collén et al., 2006b). On a longer perspective, a capacity to generate targeted red algal mutants would greatly improve the possibility to progress with the identification of defense-related genes. This, however, will require reliable transformation systems, which still need to be developed.

9. Summary

In comparison with the current knowledge on defense mechanisms in terrestrial plants, the understanding of cell-based defense in red algae is still in its infancy. Rhodophycean defense against relatively unspecific enemies such as fouling organisms may often be based upon relatively unspecific and constitutive defensive mechanisms. In contrast, red algal defenses against more specific enemies such as grazers and pathogens are often complex, enemy-aroused and based upon innate immunity. Red algae resemble terrestrial plants and animals in their basic mechanisms for pathogen recognition and signaling, which suggests that these essential cell functions arose in the sea (Potin et al., 2002; Weinberger, 2007). A good illustration of this emergence early in evolution is the dual nature of oxylipin metabolism in red algae, which have retained both animal (eicosanoids) and plant-like (octadecanoids) oxylipins. Halogenated defense metabolites play major roles in the control of microbes, fouling organisms, and grazers. Their abundancy appears as a specific adaptation of red algae to the availability of bromide in the sea. The recent development of new red algal genomic resources, such as two complete genome sequences, and of novel molecular tools, for example transcriptomics, proteomics and metabolomics, promises to be the beginning of a particularly exciting period at the cross-road between molecular and chemical Rhodophycean defense ecology.

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PART 6:
CONCLUSION AND SUMMARY

Seckbach
Israel

SUMMARY, FINAL COMMENTS AND CONCLUSIONS

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This volume is devoted to the red algae (Rhodophyta) in the genomic age. There has been a demand, with the advance in the biotechnological methods and genomic sequences, for compiling current studies of the rhodophytes. The fundamental books on red algal biology were spaced out with 17 years between each: first Kylin in 1956, then Dixon in 1973, Cole and Sheath in 1990, and now this current book (2007/09), 17–19 years later. Again, it seems like a life cycle of approximately 17 years: just an interesting observation.

The chapters in this book provide for an update of scientific work on red algae based on molecular approaches in an exciting, relatively new genomic era. Red algae comprise one important eukaryotic group for which comprehensive genetic knowledge is still under development.

Among algae, the red algae have life cycles among the complex and ecologically advanced, though asexual reproduction is common in this group. Historically, red algae have drawn attention because of their numerous interesting traits; probably to a greater extent than their green and brown counterparts. Because of their outstanding and contrasting attributes, red algae have been exploited for centuries by humans as medicine and food, and they make for ideal evolutionary and phylogenetic models.

The red algae emerged as an independent lineage some 1,500 million years ago, based on recent molecular clock analyses, which tie in with fossil evidence, thus offering unique models for studying evolution. Fossils of red algae show evidence of complex multicellularity and sexuality, making the red algae the first proven complex multicellular eukaryotes.

In the Rhodophyta there are thousands of species, making it one among the largest group. There are about 4,000–6,000 red algae species, although some estimates range from 2,500 to 20,000. Most of these groups are marine. Red algal cells do not possess flagella and centrioles. They are characterized by phycobiliproteins, some of which are found only in the red algae. They have Floridian starch as their food reserve, and the phycobilin proteins serve as accessory photosynthetic pigments, while the chloroplasts have unstacked thylakoids.

J. Seckbach and D.J. Chapman (eds.), *Red Algae in the Genomic Age*,
Cellular Origin, Life in Extreme Habitats and Astrobiology 13, 481–483
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The starring role in this volume is held by the extremophilic cyanidia algae (*Cyanidioschyzon, Cyanidium,* and *Galdieria*). These thermoacidophilic unicellular algae belong to the Rhodophytes, although they do not contain the red pigment (Phycoerythrin) and appear as blue-green (due to chlorophyll-a and phycocyanin). They can thrive in very low pH ranges and at elevated temperatures and prefer to grow under a stream of pure CO_2, while they show tolerance to salt solutions. If 2 decades ago the cyanidian group was almost unknown and obscure among the phycologists, today they serve as a good tool for genomic investigations (for more data, see Seckbach in this volume). Indeed, the first alga to have its genome sequence analyzed was *Cyanidioschyzon merolae.* The timing of this volume is fortuituous, since there has been so much progress on cyanidia since the last book on *Cyanidium* (edited by Seckbach, 1994).

Genomic approaches through genome sequencing contribute to our understanding of fundamental biological questions, including the relationships within eukaryotes and Plantae, the evolution of chloroplasts, the diversity, and evolution of advanced multicellularity. Genomic approaches also serve as valuable tools to study physiology and ecology, for example, the synthesis of economically important polysaccharides, and molecular taxonomy has benefited immensely from molecular development in the genomic era.

The 1940s and 1950s opened up an era of algal biotechnology, in which scientists discovered the urgent need for in-depth acquaintance of cellular and genomic aspects of algae, then largely unknown. Tissue culture and cell biotechnology paved the way for the genomic era in which algae were the subject of molecular approaches to understand their biology. The last 10 years or so of scientific development have clearly marked the genomic era in algae.

The red algae are thought to be one of the three groups forming the group Plantae, together with the glaucophytes and the green lineage, which may have originated from the primary endosymbiosis event between a eukaryote and a cyanobacterium (about 1,600 million years ago), thus creating the first chloroplast. In addition, there may have been extensive unidirectional horizontal transfers of genes through secondary endosymbiosis between a red alga and an unknown organism that was the ancestor to heterokonts and alveolates. Hence, there are evolutionary traces of red algae in a large portion of the eukaryotic domain. As a result of gene transfer from organellar genomes to nuclear genomes, these events are not only important for organellar genomes but are also likely to have had a significant influence on the evolution of nuclear genomes. Therefore, the key position of red algae makes knowledge of their genomes crucial to the understanding of the evolution of eukaryotes in general.

The genomic age for red algae has been part of an ongoing process probably commencing in the 1990s, when molecular tools and genome sequencing were at hand.

This volume contains 25 chapters contributed by 80 authors and coauthors who participated in the studies and their publication. These essays provide an overview of our current understanding of the genomic studies of the Rhodophytes.

The chapters cover a whole range of red algae from the primitive unicellular micro-cyanidia to the multicellular marine macroalgae. All manuscripts have been peer reviewed in order to ensure their quality. We divided this compendium into five main groupings, namely, (1) Origin and Evolution, (2) General studies of Rhdophyta, (3) Genomic and Biotechnology Investigations, (4) The Cyanidia; and (5) Biochemistry and physiology of the Red Algae.

This book covers a wide range of subjects related to red algal biology as aided by these molecular tools, from the understanding of molecular pathways aimed toward the synthesis of important molecules, biotechnological applications, phylogeny, and evolution. This is certainly a comprehensive, summary of recent advances of both unicellular and multicellular red algae.

It is hoped that the reader will benefit from this volume, providing a compendium of knowledge and thus assisting them in future investigations.

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