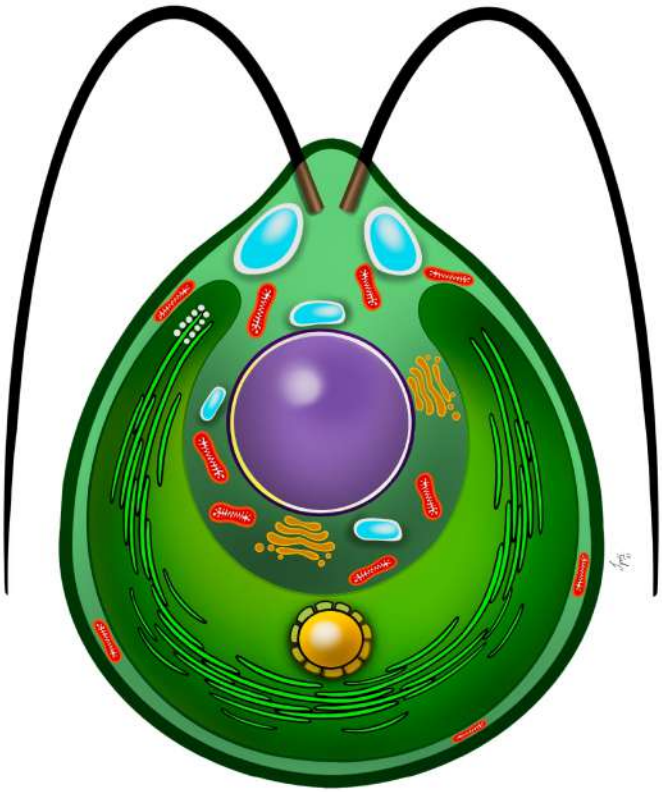
The Chloroplast of *Chlamydomonas reinhardtii*, **a Small Photosynthetic Factory**



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UEES

Cover

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2024

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Citation:
(Larrea-Álvarez, 2024)

Reference:
Larrea-Álvarez, M. (2024). The Chloroplast of *Chlamydomonas reinhardtii*, a small photosynthetic factory. Universidad Espíritu Santo - Ecuador.

Cover Page:
Universidad Espíritu Santo

Design and Publisher:
TRIBU Soluciones Integrales
Urdesa Norte Av. 2da. #315
Phone number: (593-4) 2383926
eperalta@tribuec.net

Edition:
First, April 2024

ISBN-E: 978-9978-25-250-5

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Acknowledgement

I am very grateful to Dr. Miroslava Anna □efcová for her help with editing and reviewing this manuscript.

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Prologue

The production of pharmaceutical proteins is one of the main goals of industrial biotechnology, which relies on the use of bacteria, mammalian cells, or yeast. However, other organisms have also been tested. Cyanobacteria and microalgae show high potential not only because they are photosynthetic, but also because of the bioactive compounds they produce. Still, only some species have been exploited for biotechnology. Of these species, the chlorophyte *Chlamydomonas reinhardtii* has been used to develop several molecular tools that permit genome engineering. In particular, the chloroplast is a compartment that offers many advantages. It has a small genome, with no more than a hundred genes, and homologous recombination allows targeted integration of foreign information. Several reports have documented the successful expression of recombinant proteins from the plastome of *C. reinhardtii*. The goal of this manuscript is to outline the main features that make this organelle a convenient platform for the production of valuable proteins, such as therapeutics, and along the way to review its origins and adaptations not only in *C. reinhardtii* but also in other algae.

Chapter 1

Introduction

We commonly connect the concepts of photosynthesis and carbon fixation with plants. Yet, it's important to remind ourselves of the complex processes we studied in school, which intricately linked light energy to chemical energy, culminating in sugar production. However, the abilities needed to carry out these biochemical reactions actually evolved in single-celled prokaryotes between 2.6 and 3.5 billion years ago. These cyanobacteria possessed the capability to harness sunlight energy, utilizing it to produce the essential reducing power and ATP required for converting CO_2 and water into sugar molecules. However, these reactions had a byproduct: oxygen, which gradually built up in the atmosphere, leading to the expansion of aerobic life. This increase in oxygen caused a mass extinction event as it was toxic to early anaerobic microbes. These cyanobacteria fundamentally altered the chemical makeup of the planet, and they continue to play vital ecological roles as primary producers to this day. They have formed mutualistic relationships with various organisms because their metabolic byproducts serve as a source of nutrients. For instance, certain filamentous cyanobacteria not only fix carbon but also convert atmospheric nitrogen into biologically usable forms like ammonia. Interestingly, ancient cyanobacteria were engulfed by early eukaryotes containing mitochondria in a process known as endosymbiosis. This event ultimately led to the evolution of the chloroplast, the specialized organelle responsible for sugar production in algae, plants, and some protists.

Organelles have their origins in free-living bacteria, with mitochondria descending from oxygen-consuming bacteria and chloroplasts evolving from photosynthesizing ones. In plants, chloroplasts are typically present in numerous copies, while in algae, they are usually found as a single copy. Regardless of the organism, these organelles share a common structural feature: a system of three membranes comprising inner and outer membranes that regulate the passage of small molecules and large proteins. Large compounds are facilitated through translocons, protein complexes spanning these membranes. Additionally, thylakoids are present, organized into grana and lamellae, housing complexes essential for energy generation and reducing power production. These complexes include photosystems, cytochromes, and ATP synthase. Furthermore, these structures contain photosynthetic pigments such as carotenoids, which aid in the absorption and transfer of light energy. The electrons and ATP generated during the thylakoid reactions are subsequently utilized by enzymes in the stroma to convert atmospheric CO_2 into sugars. The stroma, akin to the cytosol of the original cyanobacterium, not only houses starch granules but also contains protein and lipid bubbles known as plastoglobuli. While the primary function of the chloroplast organelle is carbon fixation, it also plays crucial roles in amino acid and nucleotide synthesis, as well as fatty acid production. With its own genome comprising genes related to photosynthesis/carbon fixation, as well as RNA and protein production, the chloroplast's genetic organization and information processing resemble those of bacteria. The nucleus assumes a pivotal role in regulating the expression of plastid genes, with key nuclear factors overseeing transcription and translation. Recently, there has been a surge in interest from both agricultural and pharmaceutical sectors in leveraging chloroplasts for engineering purposes.

Organisms that inherited chloroplasts followed distinct evolutionary paths. Glaucophytes, rhodophytes, and chlorophytes all possess primary chloroplasts originating from the original cyanobacterial cell. These organelles feature two membranes, although glaucophytes have retained a peptidoglycan layer between them. These microalgae vary in their pigment composition: while

all produce chlorophyll *a*, glaucophytes and rhodophytes also synthesize phycobiliproteins (PBP), absent in chlorophytes. Conversely, chlorophytes produce chlorophyll *b* alongside certain carotenoids. Rhodophytes typically contain chlorophyll *c*, along with orange pigments found in chlorophytes. Starch production is common among these algae, although storage locations differ. Rhodophytes and glaucophytes accumulate starch in the cytoplasm, whereas chlorophytes store it within the plastid. Later, green and red algae were engulfed by other heterotrophic eukaryotes, leading to the emergence of secondary chloroplasts. These organelles possess more than two membranes and, in some instances, remnants of the original cell membrane, nucleus, and cytoplasm. For instance, Euglenoids inherited a chloroplast derived from green algae, featuring two cyanobacterial membranes and an additional membrane associated with the heterotroph's phagosome. Starch storage in these organisms occurs outside the organelle, in the cytoplasm. Similarly, chlorarachniophytes store carbon reserves in the cytoplasm, possessing plastids inherited from chlorophytes with a four-membrane system. Vestigial eukaryotic nuclei, known as nucleomorphs, are also identifiable in some cases.

Secondary chloroplasts originating from red algae are found in various organisms. Cryptophytes possess organelles with four membranes and contain chlorophylls and phycobilins, giving them a brown-red to blue-green appearance. Haptophytes and heterokontophytes produce chlorophylls *a* and *c*, along with a distinctive carotenoid called fucoxanthin, resulting in a brown to greenish coloration due to the combination of these pigments. Similarly, dinoflagellates harbor a red algae-derived organelle producing chlorophylls *a* and *c*, as well as β-carotenes and other specific carotenoids known as xanthophylls, imparting a unique golden-brown hue. Remarkably, some photosynthetic organisms have lost their photosynthetic capabilities and evolved into full-time parasites. Their organelles, called apicoplasts, are characteristic of infamous organisms like those responsible for malaria or toxoplasmosis. Additionally, other symbiotic events have been documented. Around 100 million years ago, a cyanobiont entered into a symbiotic relationship with an amoeboid, giving rise to the photosynthetic species *Paulinella*. Moreover, it has been observed that the

ciliate *Pseudoblepharisma* contains two photosynthetic symbionts, a green alga, and a purple sulfur bacterium, although this phenomenon is still relatively new to researchers. Algae have long been utilized by humans, particularly in food culture, but their applications have expanded in recent times to include bioremediation, biofuel production, animal feed, and molecular farming. Successful engineering of algae genomes has been achieved in some cases, notably in the chlorophyte *C. reinhardtii*.

C. reinhardtii, a unicellular organism, possesses a green chloroplast containing chlorophyll *a* and *b*, alongside various carotenoids. The cup-shaped chloroplast houses thylakoids with pyrenoids and an eyespot, serving as a photoreceptive organelle crucial for phototaxis. Loaded with light-gated ion channels, the eyespot generates a membrane potential essential for flagellar movement, aiding the microalga's navigation towards optimal light and nutrient-rich environments. Occurring in both soil and aquatic habitats, *C. reinhardtii* typically inhabits upper regions where light is abundant, utilizing its swimming capability to optimize light exposure and nutrient acquisition. While primarily phototrophic, it can also engage in heterotrophic feeding. Acetate metabolism enables carbon redirection through the citric acid cycle, ultimately leading to glucose production via gluconeogenesis, an adaptation to anoxic environments with limited light. Reproduction in *C. reinhardtii* can occur both sexually and asexually, triggered by specific environmental cues related to nutrient and light availability. Vegetative haploid cells reproduce asexually, but under harsh conditions, they can fuse to form zygotes, which develop into resistant zygospores capable of surviving freezing or desiccation due to their multi-layered cell wall. Sexual reproduction enhances genetic diversity, increasing adaptability to changing environments. When favorable conditions return, dormant zygospores undergo meiosis, yielding haploid cells that resume vegetative growth. These physiological traits make *C. reinhardtii* a valuable tool in biological research, facilitating investigations into photosynthesis, flagellar movement, the cell cycle, gene expression, and genetic as well as metabolic engineering. Biotechnological advancements have enabled genome sequencing of its nuclear, mitochondrial, and chloroplast

genomes, as well as the expression of foreign proteins, broadening its utility in various scientific endeavors.

The chloroplast genome exhibits a circular DNA structure divided into four regions: two single copies and two inverted repeats. Approximately a hundred genes have been identified, with 60% encoding proteins, 30% encoding tRNAs, 6% encoding rRNAs, and the remainder associated with various functions. These genes fall into three primary groups. Firstly, genes related to photosynthesis, including those encoding components of the photosystems and the *b6f* cytochrome complex. Secondly, genes encoding machinery for replication and expression, such as DNA/RNA polymerases and ribosomes. The final group encompasses sequences linked to enzymes that contribute to the stability of these components. In chloroplasts, the flow of genetic information has retained many prokaryotic-like features inherited from cyanobacteria due to their shared ancestry. Notably, bacterial-like promoters, such as sigma^{70}-like promoters, have been identified, and a single plastid-encoded RNA polymerase is active in the organelle. Transcript degradation plays a critical role in various processes, including the removal of damaged molecules, nucleotide recycling, and gene expression regulation. Specific sequences within transcripts are essential for interaction with nucleases, thereby regulating degradation. The translational machinery of chloroplasts has evolved specific components, although counterparts for proteins found in *Escherichia coli* and spinach subunits have been identified. Translation of mRNA involves interaction between particular transcript sequences and factors encoded in the nucleus and imported from the cytoplasm. The ongoing crosstalk between nuclear and chloroplast factors is vital for organelle metabolism and functionality.

This relatively straightforward system has garnered attention from researchers interested in engineering biological systems, not only due to its advantages in culture conditions but also because of the potential for protein compartmentalization. The design of a vector is crucial for introducing genes into the plastome, ensuring successful transgene expression. Genes of interest must be flanked by sequences necessary for homologous recombination, allowing targeting to neutral sites. To induce proper transcription and

translation, transgenes must be coupled with endogenous sequences. Promoters and untranslated regions (UTRs) from photosynthetic genes are commonly utilized, along with other sequences from the transcription machinery. Selectable markers play a vital role in identifying cells that have acquired foreign genetic information. Although antibiotic resistance genes are frequently used, concerns surrounding their use have led to the development of alternative selection methods. These include photosynthesis restoration, arginine synthesis, and phosphate availability, among others. Advancements in reporter genes have further enhanced chloroplast engineering by encoding detectable phenotypes. Successful expression and detection of bioluminescent proteins, such as luciferases, have been achieved. However, the expression of fluorescent proteins has been less successful due to challenges in effectively detecting fluorescence from chloroplast-derived proteins. Introducing the vector into the chloroplast for integration into the genome via homologous recombination can be achieved through various methods, including electroporation, biolistics, or agitation with glass beads.

These methodologies have facilitated the integration and expression of diverse proteins sourced from various origins. While most are single-subunit proteins encoded by a single gene, there have been reports of multiple engineering of the plastome. Although the majority of recombinant proteins are soluble and located in the stroma, some are membrane-associated. Over a hundred proteins have been successfully produced in the chloroplast, with approximately 40 possessing therapeutic properties. Notable examples include vaccines, monoclonal antibodies, antibacterials, and bioactive peptides. These achievements demonstrate the feasibility of folding and accumulating foreign proteins within the chloroplast. However, the potential of this compartment as a platform for recombinant expression could be further enhanced through the application of synthetic biology principles. The development of libraries and one-step assembly techniques would help improve the yield of recombinant proteins. Genetic engineering of the chloroplast of *C. reinhardtii* is commonplace in laboratories and companies worldwide, with future advancements focusing on metabolic engineering. Indeed, the technologies

established in *C. reinhardtii* could serve as a basis for transforming the chloroplasts of other commercially relevant microalgae, such as *Dunaliella* or *Chlorella*, thereby amplifying the production of valuable products in photosynthetic microbes.

This manuscript aims to provide a concise overview of the chloroplast of *C. reinhardtii* within both evolutionary and technological contexts. It seeks to integrate the organelle's origins and development with its utilization as a platform for recombinant protein expression, while also exploring intriguing aspects of algae, a group of ecologically significant organisms.

Chapter 2

Cyanobacteria, the oldest photosynthesizer

The emergence of single-celled photosynthetic microbes had profound implications for the history of our planet. Not only did it lead to a significant extinction event among early microorganisms, but it also set the stage for the evolution of complex life on Earth. These ancient cyanobacteria, appearing around 3.5 billion years ago, played a pivotal role by producing oxygen as a byproduct of their metabolic processes. This oxygen, once released into the atmosphere, forever altered its composition. During this period, nitrogen (N_2), carbon dioxide (CO_2), and methane (CH_4) were predominant, with oxygen (O_2) mostly bound in molecules like water (H_2O). Anaerobic microbes thrived in this environment, obtaining energy from various available molecules. However, between 2.6 and 3.5 billion years ago, certain microbial species evolved the ability to perform photosynthesis. Through specialized structures in their membranes, they could harness sunlight energy to produce sugars and oxygen gas from CO_2 and water.

These early photosynthetic microbes, now recognized as ancestors of cyanobacteria, gained a significant evolutionary advantage. Their ability to generate energy from abundant raw materials spurred a population explosion. As atmospheric oxygen levels began to rise, it initially reacted with iron before exceeding the absorption capacity. The resultant oxygen-rich atmosphere proved toxic to many anaerobic organisms, leading to a mass extinction event around 2.5 billion years ago, known as the oxygen catastrophe or great oxygenation event. Life eventually adapted to these conditions, with aerobic

microorganisms utilizing oxygen for metabolizing sugars or fats. Oxygen levels fluctuated over time until stabilizing at the current 21%. The capacity to utilize oxygen for energy acquisition facilitated the diversification and evolution of more complex life forms. Cyanobacteria further influenced this narrative through endosymbiotic events, where ancient versions were engulfed by early eukaryotic cells, contributing to the development of specialized organelles like chloroplasts, essential for energy production in plant cells.

Photosynthesis, often referred to as oxygenic photosynthesis, involves the splitting of water into electrons, protons, and oxygen. The chemical energy generated is stored in simple sugars, serving as the building blocks for polysaccharides like starch or cellulose. Organisms capable of oxygenic photosynthesis, known as photoautotrophs, can produce their own food using light. They are vital for sustaining complex life on Earth as they not only generate biological energy but also help maintain the balance of oxygen levels in the atmosphere. Ecologically, they are recognized as primary producers, synthesizing organic compounds that form the foundation of the food chain.

While we primarily discuss oxygenic photosynthesis, early photosynthetic organisms likely used different electron donors aside from water. Some utilized molecules such as hydrogen, sulfur, or various organic acids, processes collectively termed anoxygenic photosynthesis. In any case, cyanobacteria played a pivotal role in oxygenating the atmosphere and facilitating the development and expansion of aerobic life. Considered the oldest photosynthetic organisms, all other photosynthesizers are believed to have descended from them. The name “cyanobacteria” derives from the color cyan, and they are often mistakenly referred to as blue-green algae. Cyanobacteria encompass a diverse group of gram-negative photoautotrophic prokaryotes, varying from unicellular to filamentous to colonial aggregates (Figure 1). Certain cyanobacteria, particularly filamentous forms, possess the ability to fix both carbon and nitrogen from the atmosphere. This occurs in specialized cells known as heterocysts, where N_2 gas is converted into ammonia and subsequently into other forms usable by plants for protein and nucleic acid synthesis. Other specialized cells, such as akinetes for dormancy

and hormogonia for reproduction, are also recognized. Ubiquitous and significant in marine primary production, cyanobacteria play a crucial role in various ecosystems.

Cyanobacteria have attracted considerable attention in the realm of human nutrition due to their ability to synthesize various high-value products. These include proteins, vitamins, minerals, amino acids, pigments, antioxidants, and polyunsaturated fatty acids. Moreover, these microorganisms produce polysaccharides with immunomodulatory, anti-inflammatory, anti-tumoral, antibacterial, and antiviral properties. Among the species utilized for these purposes, *Arthrospira platensis* and *Aphanizomenon flos-aquae* stand out as popular choices. *A. platensis*, commonly known as *Spirulina*, has been marketed extensively as a health supplement, available in tablet or powder form (Figure 2). *A. flos-aquae*, on the other hand, has been employed for developing products with neuromodulatory and anti-inflammatory properties. Additionally, the photosynthetic activity of cyanobacterial cells holds significant promise for generating renewable energy. By harnessing sunlight and converting it into electrical signals, the photosynthetic machinery of cyanobacteria can be coupled with chemical mediators to pass electrons to external electrodes. Current efforts are focused on exploiting this capability to produce biofuels, including diesel, jet fuel, and gasoline.

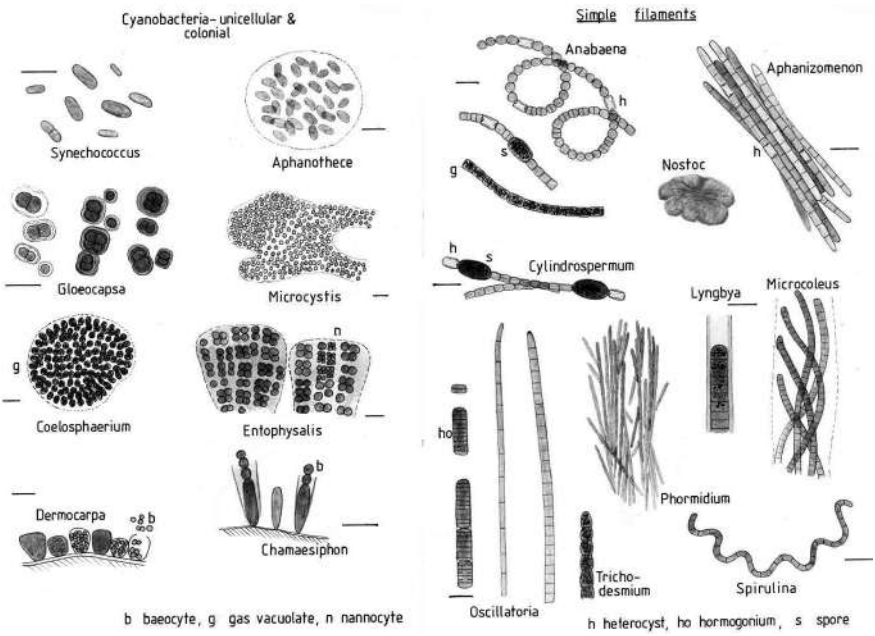


Figure 1. Shapes and forms of cyanobacteria. Source: File:Cyanobacteriaunicellularandcolonial020.jpg. (2022, July 12). *Wikimedia Commons*. Retrieved August 18, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Cyanobacteriaunicellularandcolonial020.jpg&oldid=673865751; File:Simplefilaments022.jpg. (2022, October 24). *Wikimedia Commons*. Retrieved August 18, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Simplefilaments022.jpg&oldid=698735679. (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).

Figure 2. Spirulina powder and tablets. Source: File:Spirulina.jpg. (2020, October 4). *Wikimedia Commons*. Retrieved August 22, 2023 from https://commons.wikimedia. org/w/index.php?title=File:Spirulina jpg&oldid=480539813; File:Spirulina tablets.jpg. (2023, June 10). *Wikimedia Commons*. Retrieved August 22, 2023 from https://commons wikimedia.org/w/index.php?title=File:Spirulina_tablets.jpg&oldid=772955198 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org licenses/by-sa/4.0/deed.en).

In the process of photosynthesis, everything starts with the absorption of photons, which causes a specific chlorophyll molecule to lose an electron. This electron is then transferred to other molecules, initiating an electron transport chain that leads to the production of reducing power. Simultaneously, the movement of electrons generates a proton gradient across the membrane, which is utilized by ATP synthase to produce ATP. The lost electron from the chlorophyll molecule is replenished through the splitting of water molecules via photolysis, a process driven by sunlight energy, occurring in the "oxygen-evolving complex." The energy required to eject electrons from these specialized chlorophylls (P680nm and P700nm, named after their absorption wavelengths) is captured by other chlorophylls and accessory pigments, such as carotenoids. These components are organized into complexes known as photosystems, specifically Photosystem I (PSI) and Photosystem II (PSII). Within PSI, the excited electron is further energized and transferred to other acceptor molecules, ultimately leading to the reduction of $NADP^+$ to NADPH, as illustrated in the upper panel of Figure 3.

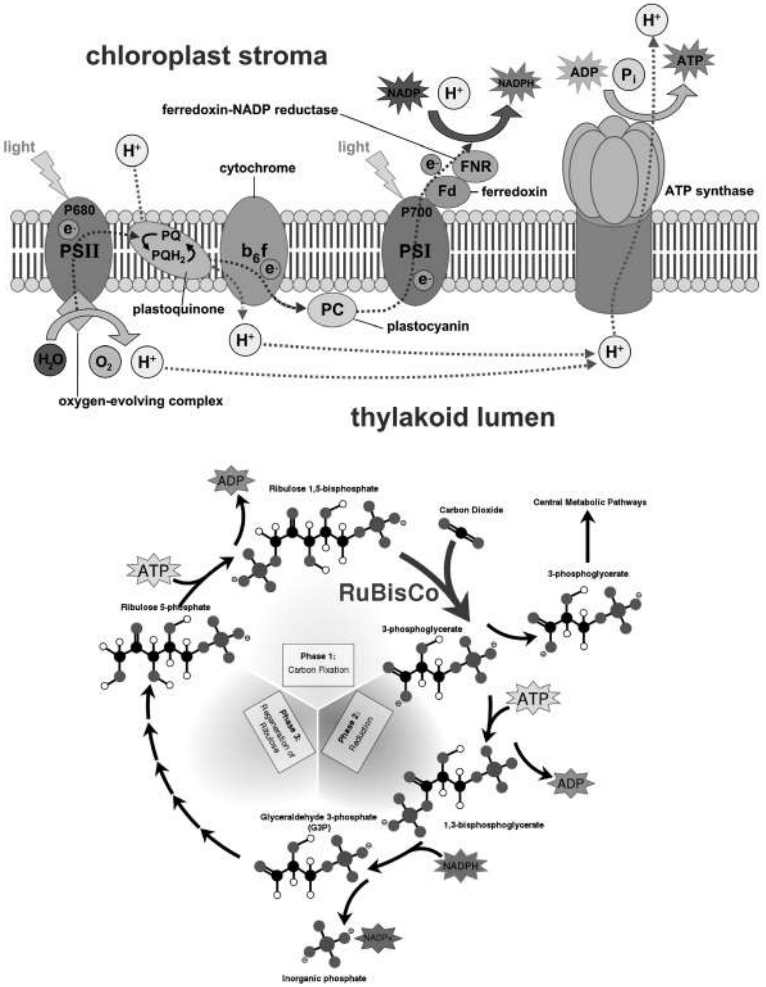


Figure 3. Reactions in the thylakoid membranes and the Calvin cycle. Source: File:Thylakoid membrane 3.svg. (2023, August 28). *Wikimedia Commons*. Retrieved August 30, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Thylakoid_membrane_3.svg&oldid=796575813; File:Calvin-cycle4.svg. (2023, August 28). *Wikimedia Commons*. Retrieved August 30, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Calvin-cycle4.svg&oldid=796573270 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).

Following the synthesis of NADPH and ATP, these molecules are utilized in the Calvin cycle to produce three-carbon sugars. Through a series of enzymatic reactions, these molecules are combined to form six-carbon sugars, serving as monomers for larger polysaccharides. Carbon fixation, a crucial step in this process, is facilitated by the enzyme RUBISCO (ribulose 1,5-bisphosphate carboxylase oxygenase), which captures carbon dioxide from the atmosphere and combines it with ribulose 1,5-bisphosphate, yielding two molecules of 3-phosphoglycerate. These molecules are then phosphorylated and reduced to glyceraldehyde 3-phosphate. While a portion is used to regenerate the acceptor and sustain the cycle, the remaining triose phosphates condense to form hexoses, serving as precursors for sucrose, starch, and cellulose synthesis, as depicted in the lower panel of Figure 3.

In cyanobacteria, photosynthesis occurs within specialized flattened sacs called thylakoids, which are distinct compartments from the plasma membrane. The machinery necessary for ATP and reducing power production is embedded within these thylakoid membranes. Additionally, water-soluble proteins, including phycobiliproteins, are attached to these membranes. These phycobiliproteins harvest light energy and transfer it to chlorophylls, imparting the characteristic blue-green color to cyanobacteria (Figure 4).

ATP and reducing power generated within the thylakoids are utilized for carbon fixation into sugars. Cyanobacteria accomplish this within specialized compartments known as carboxysomes, which are polyhedral protein shells containing numerous copies of the RUBISCO enzyme. These structures facilitate the concentration of CO_2 around RUBISCO, minimizing oxygen levels around the enzyme (Figure 4). It's important to note that oxygenation of ribulose bisphosphates by RUBISCO can lead to the loss of energy for carbon fixation, a process termed photorespiration.

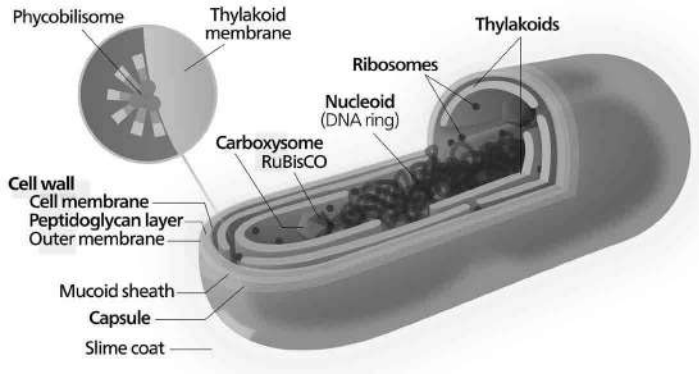


Figure 4. A cyanobacterial cell. Source: File:*Cyanobacterium*.svg. (2023, August 28). *Wikimedia Commons*. Retrieved August 31, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Cyanobacterium.svg&oldid=796574272 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en.).

In cyanobacteria, genetic material is typically organized within one or two chromosomes, along with plasmids and other genetic elements. The majority of information required for constructing the complex cellular machinery is encoded within the chromosomal genome. Plasmids, although less prevalent, have been associated with various functions including antibiotic resistance, resistance to heavy metals, adaptation to high salt concentrations, and toxin production. A set of conserved genes has been identified within cyanobacterial genomes, predominantly comprising sequences encoding complex proteins and fundamental biochemical pathways. These pathways encompass essential cellular processes such as photosynthesis, carbon fixation, respiration, DNA replication, and gene expression. Cyanobacteria engaged in symbiotic relationships with other organisms are referred to as cyanobionts. These relationships can involve cyanobionts residing either inside or outside the host organism, with mutual exchange of signals being vital for their mutual thriving. Cyanobionts have formed symbiotic associations with a diverse array of organisms including plants, diatoms, sponges, unicellular organisms, and multicellular parasites. These symbiotic interactions are primarily mutualistic,

where cyanobionts provide nutrients to the host while benefiting from a specialized niche provided by the host organism, as depicted in Figure 5.

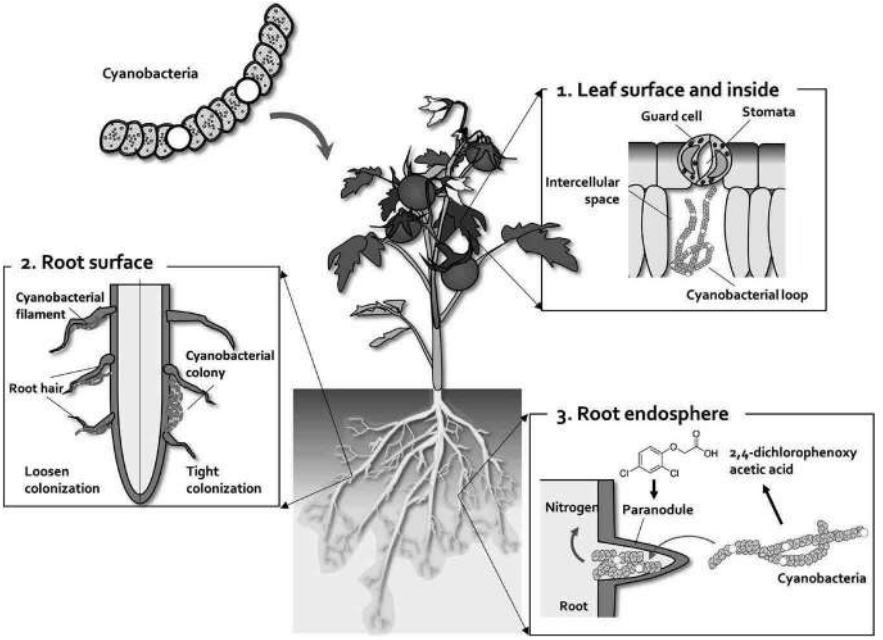


Figure 5. Interactions between cyanobacteria and plants. Source: File:Leaf and root colonization by cyanobacteria.jpg. (2021, August 5). *Wikimedia Commons*. Retrieved September 1, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Leaf_and_root_colonization_by_cyanobacteria.jpg&oldid=578572843 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en.).

At a certain point in evolutionary history, a member of the archaea group, an ancient group of bacteria, engulfed a bacterial cell, giving rise to the ancestor of mitochondria. Subsequently, this newly formed eukaryotic cell engulfed photosynthesizing cyanobacteria. This process of endosymbiosis led to the development of mutualistic relationships between the engulfed and engulfing cells. As a result, these microbial guests opted to reside permanently within their hosts. These endosymbionts are now widely accepted as the ancestors of the organelles responsible for respiration and photosynthesis. This fascinating evolutionary journey forms the subject of the subsequent chapter.

Chapter 3

From a photosynthesizing cell to a specialized organelle

After indulging in a delicious carbohydrate-rich meal, one often experiences a surge in energy—a direct result of the nutrients synthesized by the plants from which our food originated, such as corn or wheat. The process behind this energy production is photosynthesis, where sunlight is utilized to convert water and carbon dioxide into energy-rich sugars. This remarkable process unfolds in two main steps: first, the generation of chemical energy (ATP) and reducing power (electrons), and second, the Calvin cycle, which fixes carbon dioxide into sugars. This photosynthetic mechanism stands as one of the most sustainable production frameworks to have evolved. In plants, photosynthesis takes place within specialized organelles called chloroplasts, though we've learned that these traits trace back to ancient prokaryotes, and that cyanobacteria and algae are also proficient photosynthetic organisms. Over two billion years ago, prokaryotes were the dominant life forms, exhibiting a rich diversity of metabolic capabilities. Some were photosynthetic, while others utilized oxygen for ATP production, and still others were larger and capable of engulfing smaller oxygen-consuming bacteria. Rather than digesting these engulfed bacteria, some prokaryotes formed symbiotic relationships with them. These new entities are believed to be the ancestors of heterotrophic eukaryotes, some of which later engulfed photosynthetic bacterial cells, giving rise to autotrophic eukaryotes.

We regard the oxygen-consuming bacteria as the ancestors of mitochondria, and the bacteria capable of harnessing sunlight for food production as the precursors of chloroplasts. Mitochondria are presumed to have evolved before chloroplasts, as all eukaryotic cells contain mitochondria, while only a subset possess chloroplasts.

Prokaryotes, believed to have emerged as early as four billion years ago, encompass microorganisms like bacteria and archaea, characterized by their simplicity and possession of circular genomes that float freely within the cell. In contrast, eukaryotes are larger and more complex, featuring their primary genome enclosed within a membrane-bound nucleus. All organisms visible to the naked eye or under basic microscopes are eukaryotes; their emergence around two billion years ago marked a milestone that fundamentally altered the biological landscape. In the mid-20th century, researchers began formulating an explanation for the evolution of eukaryotes. According to this theory, certain bacteria began residing within larger bacterial cells, leading to a symbiotic relationship where both entities benefited. Over time, this symbiosis became inseparable, culminating in the integration of the smaller bacterium into the larger host cell, resulting in a single, unified organism. This merger gave rise to specialized organelles, within which crucial metabolic processes were compartmentalized. Organelles can be categorized based on their structure into two main types: single membrane-bound organelles, such as the vacuole, lysosome, Golgi apparatus, and endoplasmic reticulum, and double membrane-bound organelles, including the nucleus, mitochondria, and chloroplasts (Figure 6).

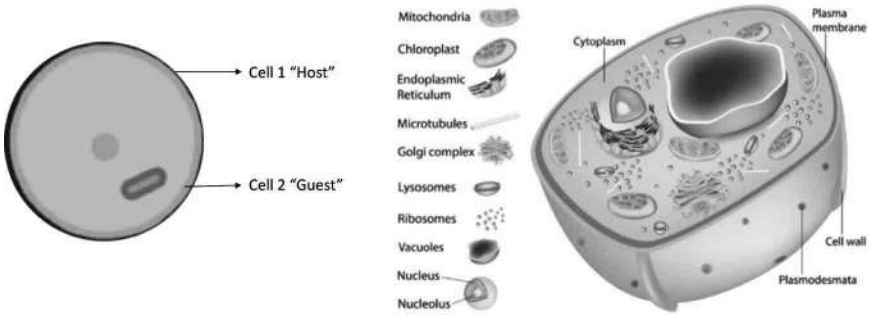


Figure 6. Development of eukaryotic cells. Adapted from: "*Cell Organelles: Endoplasmic Reticulum and Golgi Body*". (2023, January 25). EMBIBE. Retrieved September 7, 2023 from https://www.embibe.com/exams/cell-organelles/.

All eukaryotic cells possess mitochondria, often referred to as the "powerhouse of the cell" due to their role in synthesizing the energy-storing molecule ATP. However, only certain eukaryotes also contain chloroplasts, which convert sunlight into sugars. Remarkably, both chloroplasts and mitochondria exhibit striking similarities to bacteria. They replicate via binary fission, a process akin to bacterial reproduction, wherein new organelles arise from existing ones. Furthermore, these structures are encased in double membranes, a characteristic typical of bacterial cells. Although these resemblances were observed as early as the 20th century, many biologists remained skeptical. It wasn't until the 1960s and 1970s, with the work of Lynn Margulis, an American evolutionary biologist, that this idea gained traction. By the late 1980s, it had become widely accepted within the scientific community. Subsequent genetic research revealed that both mitochondria and chloroplasts possess their own DNA, distinct from the nuclear DNA. Genome analyses further demonstrated that the mitochondrial genome shares similarities with that of *Rickettsia*, an intracellular bacterium, while the chloroplast genome closely resembles that of cyanobacteria (Figure 7). The genetic and anatomical parallels provide compelling evidence supporting the theory that these organelles originated from ancient free-living bacteria. Remarkably, such symbiotic events occurred not just once, but at least three times in evolutionary history. The first event, around 2.4 billion years ago, gave rise to mitochondria and ultimately led to

the emergence of the entire eukaryotic lineage. Subsequently, approximately 1.6 billion years ago, cyanobacteria established residence within certain eukaryotic cells, resulting in primary chloroplasts, which facilitated the development of plant life. Another event occurred approximately 450 million years ago, wherein a single-celled eukaryote engulfed cells containing primary chloroplasts. Finally, some eukaryotes engulfed others containing chloroplasts derived from red or green algae, a topic explored further in Chapter 4 (Figure 8).

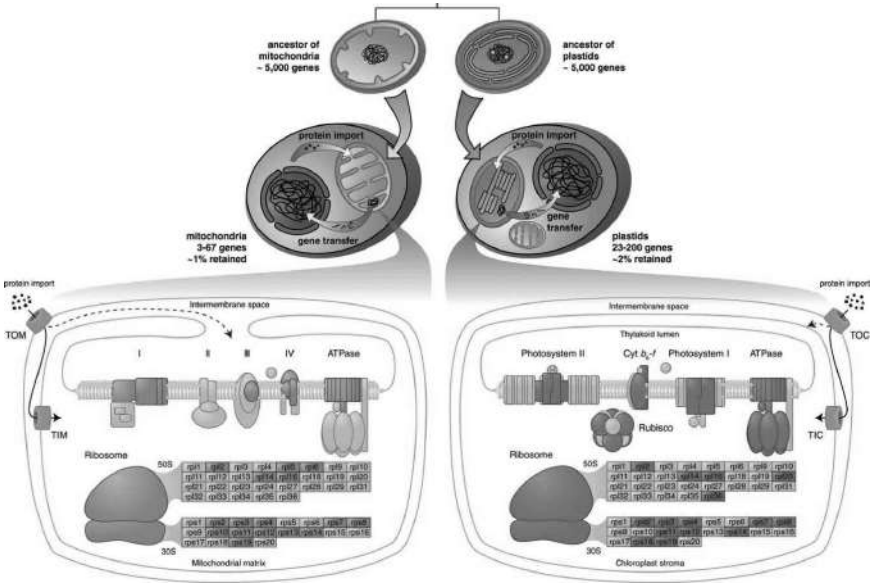


Figure 7. Origins of mitochondria and plastids. Source: "Massively Convergent Evolution for Ribosomal Protein Gene Content in Plastid and Mitochondrial Genomes" by U. G. Maier, S. Zauner, C. Woehle, K. Bolte, F. Hempel, J. F. Allen, W. F. Martin, 2013, *Genome Biology and Evolution*, 5, pp. 2318-2329.

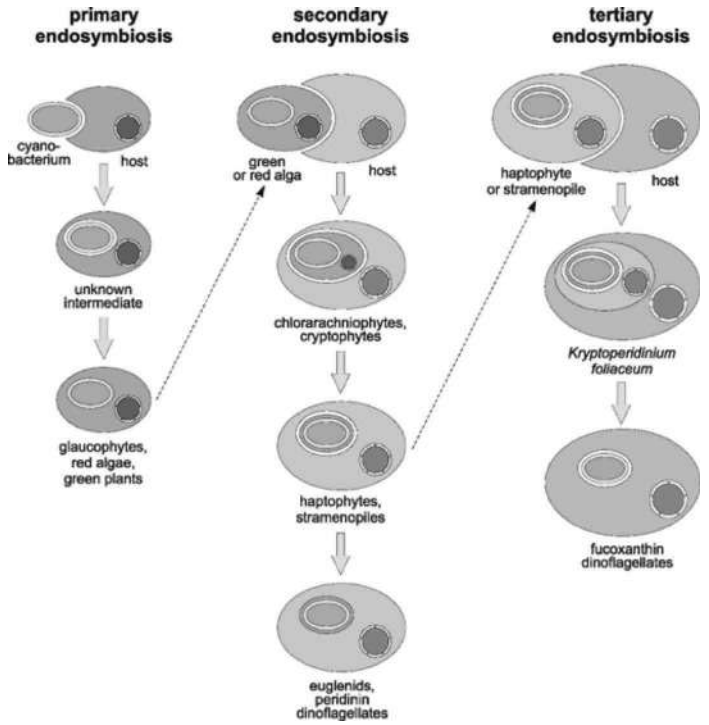


Figure 8. Endosymbiotic events associated with photosynthesis. Source: "Did Trypanosomatid Parasites Contain a Eukaryotic Alga–Derived Plastid in Their Evolutionary Past?" by A. Bodył, P. Mackiewicz, R. Milanowski, 2010, *Journal of Parasitology*, 96, pp. 465-475.

Indeed, the early endosymbiotic event between a eukaryote and a cyanobacterium led to the formation of three distinct groups: glaucophytes, rhodophytes, and chlorophytes, each harboring primary chloroplasts with unique characteristics. These variations in chloroplast morphology are evident across different organisms. For instance, in land plants, chloroplasts are typically lens-shaped and exist in numerous copies within a single cell. Conversely, algae display a diverse array of chloroplast shapes, with many species having only one chloroplast per cell. The shape of chloroplasts varies greatly among algae species. For example, in *Oedogonium*, they are shaped like a net, resembling a cup in *Chlamydomonas*, forming a ribbon-like spiral in *Spirogyra*, and appearing as twisted bands in *Sirogonium* (Figure 9). Despite

these differences in shape, all chloroplasts share common structural features, including three systems of membranes: the thylakoid, outer membrane, and inner membrane (Figure 10). The outer membrane of chloroplasts is semiporous, allowing the passage of small molecules and ions while restricting the entry of large proteins. Consequently, polypeptides synthesized in the cell's nucleus but required within the chloroplast must be transported across the outer membrane via protein complexes known as translocons. One such complex, the TOC complex (translocon on the outer chloroplast membrane), facilitates this process. Between the outer and inner membranes lies an intermembrane space. Some algae, such as glaucophytes, retain a peptidoglycan wall between their membranes, a vestige of their cyanobacterial ancestry. However, this wall has been lost in other photosynthetic eukaryotes, leaving an empty space between the membranes. The inner membrane of the chloroplast borders the stroma and regulates the movement of materials into and out of the organelle. Polypeptides that have traversed the TOC complex must pass through another protein complex, the TIC complex (translocon on the inner chloroplast membrane), to enter the stroma. These intricate membrane systems play essential roles in maintaining the functionality of chloroplasts and orchestrating various cellular processes related to photosynthesis and metabolism.

The thylakoid system within chloroplasts comprises interconnected sacs that house the machinery necessary for producing NADPH and ATP (Figure 10). Within this system, grana are stacks of flattened membranes, with each granum typically containing around 100 thylakoids. These thylakoids are surrounded by helical stromal membranes called lamellae. Grana primarily contain photosystem II (PSII), while photosystem I (PSI) and ATP synthase are predominantly found in the lamellae. Embedded within the thylakoid membranes are various photosynthetic pigments capable of absorbing and transferring light energy (Figure 10). These pigments contribute to the distinct colors associated with chloroplasts. Among the most prominent pigments are chlorophylls, with chlorophyll *a* being the primary pigment found in both cyanobacteria and chloroplasts. Chlorophyll *a* absorbs light primarily at wavelengths of 430 and 662 nm. In addition to this pigment,

other chlorophyll types have been identified, including chlorophyll *b*, *c*, *d*, and *f*. Chlorophyll *b* is a green pigment primarily found in plants, green algae, and some secondary chloroplasts, absorbing light at wavelengths of 453 and 642 nm. Chlorophyll *c*, on the other hand, is a blue-green pigment detected in certain algae such as diatoms and dinoflagellates, absorbing light in the 447–452 nm region. Cyanobacteria also synthesize chlorophyll *d* and *f*, with chlorophyll *d* absorbing light at 710 nm and chlorophyll *f* absorbing light at 707 and 800 nm. Carotenoids, including xanthophyll, α-carotene, and β-carotene, are yellow-orange pigments present in photosystems that aid in the transfer and dissipation of excess energy. Similar to chlorophyll *a*, β-carotene is widespread in chloroplasts and absorbs light at wavelengths of 450 and 475 nm. Phycobilins are another group of photosynthetic pigments associated with cyanobacteria and some algal chloroplasts. They can exhibit red (phycoerythrobilin), orange (phycourobilin), or blue (phycocyanobilin, phycoviolobilin) colors and are sometimes organized into complexes known as phycobilisomes. These pigments absorb light in the range of 500 to 650 nm. Further exploration of phycobilins will be discussed in detail in the subsequent chapter.

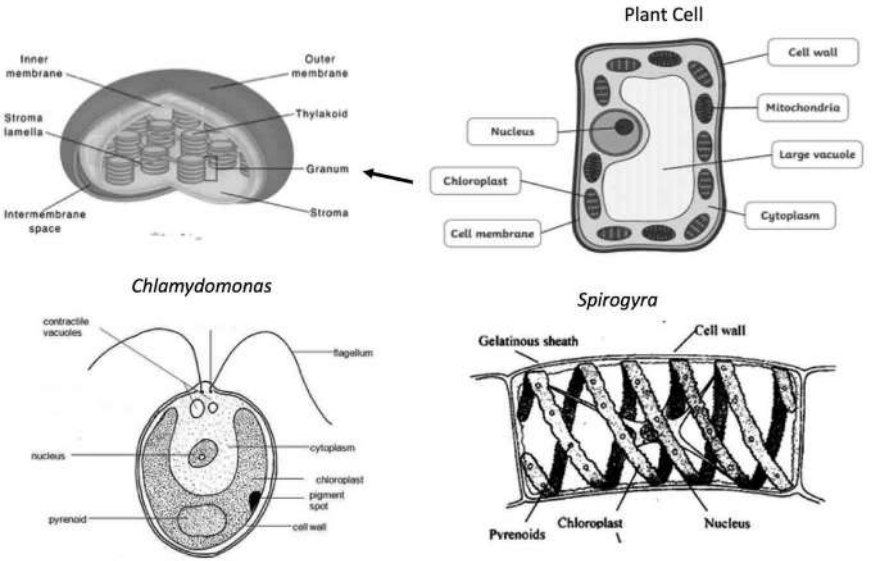


Figure 9. Chloroplast shapes. Adapted from: "*Chloroplast*". (2020, July 15). SCIENCE FACTS. Retrieved September 8, 2023 from https://www.sciencefacts.net/chloroplast.html; "*Plant cell*". (n.d.). TWINKL. Retrieved September 10, 2023 from https://www.twinkl.com.au/teaching-wiki/plant-cell; "*Chlamydomonas*". (2022, August 23). ALCHETRON. Retrieved September 10, 2023 from https://alchetron. com/Chlamydomonas#chlamydomonas-ba6698db-5dcb-406c-9da3-622eeea9366resize-750.jpeg; "*Spirogyra*". (n.d.). STUDY SOLUTION. Retrieved September 9, 2023 from https://istudy.pk/spirogyra/.

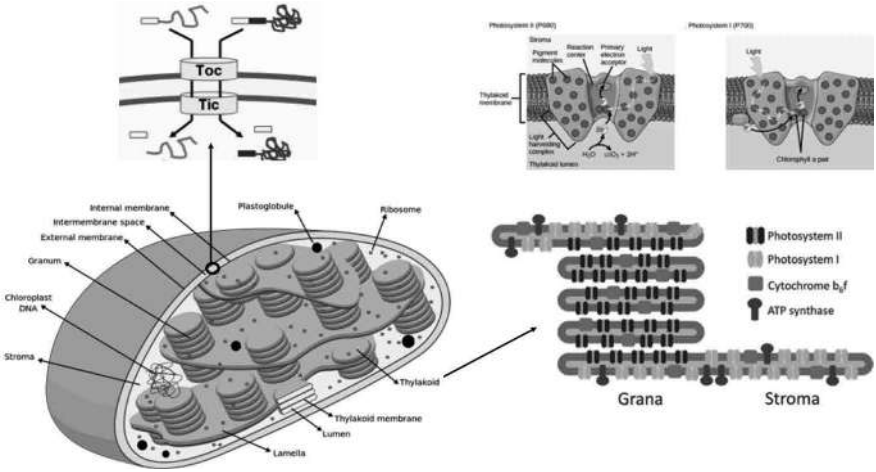


Figure 10. Structure of the chloroplast. Adapted from: "Thylakoid Direct Photobioelectrocatalysis: Utilizing Stroma Thylakoids to Improve Bio-solar Cell Performance" by M. Rasmussen, S. D. Minteer, 2014, *Physical Chemistry Chemical Physics*, 16, pp. 17327-17331; "*Photosynthesis*". (n.d.). LUMEN LEARNING. Retrieved September 8, 2023 from https://courses.lumenlearning.com/suny-wmopenbiology1/chapter/photosynthesis/; File:Thylakoid targeting.png. (2022, April 29). *Wikimedia Commons*. Retrieved September 10, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Thylakoid_targeting.png&oldid=652625933; File:Scheme Chloroplast-en.svg. (2020, September 8*). Wikimedia Commons*. Retrieved September 10, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Scheme_Chloroplast-en.svg&oldid=451823757(These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en.).

The stroma, corresponding to the cytosol of the ancestral cyanobacterium, constitutes a protein-rich, alkaline fluid housing numerous essential components (Figure 10). Notably, this milieu hosts enzymes crucial for the Calvin cycle, where carbon dioxide is converted into 3-carbon sugars. Subsequently, these sugars are condensed into hexoses, primarily glucose. Glucose can follow two metabolic pathways: Firstly, it may undergo uracil diphosphate (UDP) tagging to form UDP-glucose, a precursor for sucrose (common table sugar). Secondly, glucose can be phosphorylated to yield glucose-1-phosphate, which

is then converted into ADP-glucose, a precursor for starch synthesis (Figure 11). Starch, a polymer of glucose molecules linked by glycosidic bonds, accumulates within the stroma in the form of starch granules. These granules exhibit dynamic growth under sunlight exposure and reduction in its absence, serving as energy reserves to sustain cellular respiration. Additionally, spherical lipid-protein bubbles encased by a lipid monolayer, known as plastoglobuli, have been identified. Plastoglobuli house enzymes and structural proteins associated with lipid synthesis, containing a diverse array of lipids including chlorophylls, carotenoids, vitamin E, and plastoquinone—integral to the electron transport chain. These structures emerge either from the formation of bubbles in the lipid bilayer of thylakoids or from the growth of pre-existing plastoglobuli.

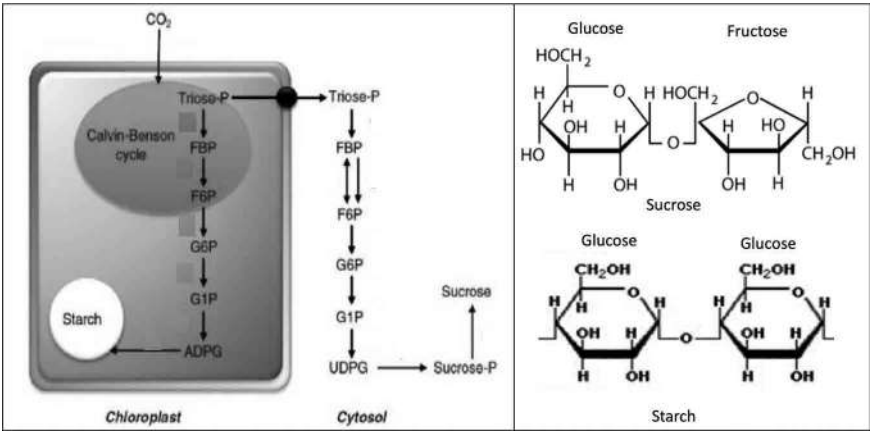


Figure 11. Starch and sucrose synthesis. Adapted from: "Starch Biosynthesis, its Regulation and Biotechnological Approaches to Improve Crop Yields" by A. Bahaji, J. Li, Á. M. Sánchez-López, E. Baroja-Fernández, F. J. Muñoz, M. Ovecka, J. Pozueta-Romero, 2014, *Biotechnology Advances*, 32, pp. 87-106; "*Carbohydrate Metabolism*". (n.d.). NYU LANGONE HEALTH. Retrieved September 11, 2023 from http://education.med.nyu.edu/mbm/carbohydrates/digestionAbsorption.shtml.

Chloroplasts, a specialized type of plastid, are primarily recognized for their role in photosynthesis. However, they also perform other vital functions. Within the stroma, amino acids are synthesized, and the production of nitrogenous bases (purines and pyrimidines) for DNA and RNA takes place.

Furthermore, complex lipid synthesis occurs within chloroplasts, where fatty acids are synthesized from acetyl-CoA through enzymatic reactions. The extension of fatty acids involves condensation, reduction, and dehydration processes. Maintaining a pH of approximately 8 in the chloroplast is crucial for optimal functioning of the Calvin cycle; deviations from this pH can disrupt metabolic reactions, which chloroplasts mitigate by removing cations (e.g., K^+, H^+) via pumps.

In addition to chloroplasts, various types of plastids exist, each with specialized functions depending on the organism. For example, in plants, plastids differentiate from proplastids and include chromoplasts (for pigment synthesis and storage), leucoplasts (colorless plastids involved in terpene production), amyloplasts (for starch storage), and proteinoplasts (for tannins and polyphenols). Algal plastids include rhodoplasts (in red algae) and muroplasts (in glaucophytes), each with unique characteristics such as red chromatophores or a peptidoglycan layer. Moreover, in certain protists like the Apicomplexa, non-photosynthetic plastids called apicoplasts are involved in fatty acid, isoprenoid, and heme synthesis, making them potential targets for drug development against parasites such as *Plasmodium falciparum* and *Toxoplasma gondii*.

Similar to mitochondria, chloroplasts are typically inherited from a single parent, although biparental inheritance has been observed in some flowering plants. Chloroplasts possess semi-autonomous genetic systems, including their own DNA (cpDNA) and machinery for transcription and translation. The chloroplast genome, or plastome, is usually circular, approximately 170,000 base pairs long, with a long single copy (LSC) and a short single copy (SSC) section separated by two inverted repeats. Plastomes typically encode genes for rRNAs, tRNAs, ribosomal proteins, subunits of RNA polymerase complex, photosynthetic thylakoid proteins, and the large subunit of RUBISCO (Figure 12) (Table 1).

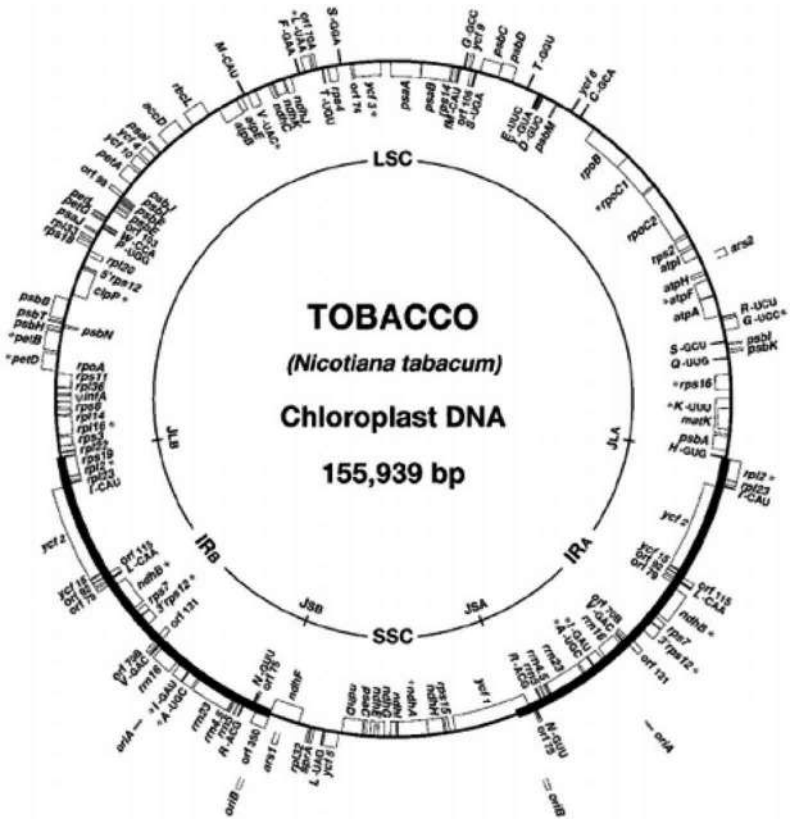


Figure 12. Tobacco chloroplast genome. Source: "Updated Gene Map of Tobacco Chloroplast DNA" by T. Wakasugi, M. Sugita, T. Tsudzuki, M. Sugiura, 1998, *Plant Molecular Biology Reporter*, 16, p. 231.

Table 1. *Genes encoded in the plastome*

GENETIC SYSTEM				PHOTOSYNTHETIC APPARATUS	
Genes	**Products**	**Genes**	**Products**	**Genes**	**Products**
rrn23	23S rRNA	*rps3*	30S r-protein CS3	*rbcL*	RuBisCO large subunit
rrn16	16S rRNA	*rps4*	30S r-protein CS4	*psaA*	PS I P700 apoprotein A1
rrn5	5S rRNA	*rps7*	30S r-protein CS7	*psaB*	PS I P700 apoprotein A2
rrn4.5	4.5S rRNA	*rps8*	30S r-protein CS8	*psaC*	PS I subunit VII
trnR-ACG	Arg-tRNA(ACG)	*rps11*	30S r-protein CS11	*psaI*	PS I 4 kDa protein
trnR-UCU	Arg-tRNA(UCU)	*rps14*	30S r-protein CS14	*psaJ*	PS I 5 kDa protein
trnR-CCG	Arg-tRNA(CCG)	*rps15*	30S r-protein CS15	*psaM*	PS I M-protein
trnN-GUU	Asn-tRNA(GUU)	*rps18*	30S r-protein CS18	*ycf4* (ORF184)	(assembly/stability)
trnD-GUC	Asp-tRNA(GUC)	*rps19*	30S r-protein CS19	*psbA*	PS II D1-protein
trnC-GCA	Cys-tRNA(GCA)	*rpl14*	50S r-protein CL14	*psbB*	PS II 47 kDa protein
trnQ-UUG	Gln-tRNA(UUG)	*rpl20*	50S r-protein CL20	*psbC*	PS II 43 kDa protein
trnE-UUC	Glu-tRNA(UUC)	*rpl21*	50S r-protein CL21	*psbD*	PS II D2-protein
trnG-GCC	Gly-tRNA(GCC)	*rpl22*	50S r-protein CL22	*psbE*	PS II cytochrome b559 (8 kDa)
trnH-GUG	His-tRNA(GUG)	*rpl23*	50S r-protein CL23	*psbF*	PS II cytochrome b559 (4 kDa)
trnI-CAU	Ile-tRNA(CAU)	*rpl32*	50S r-protein CL32	*psbH*	PS II 10 kDa phosphoprotein
trnL-CAA	Leu-tRNA(CAA)	*rpl33*	50S r-protein CL33	*psbI*	PS II I-protein
trnL-UAG	Leu-tRNA(UAG)	*rpl36*	50S r-protein CL36	*psbJ*	PS II J-protein
trnfM-CAU	fMet-tRNA(CAU)	*rpoA*	RNA polymerase subunit α	*psbK*	PS II K-protein
trnM-CAU	Met-tRNA(CAU)	*rpoB*	RNA polymerase subunit β	*psbL*	PS II L-protein
trnF-GAA	Phe-tRNA(GAA)	*rpoC2*	RNA polymerase subunit β''	*psbM*	PS II M-protein
trnP-UGG	Pro-tRNA(UGG)	*infA*	initiation factor 1	*psbN*	PS II N-protein
trnP-GGG	Pro-tRNA(GGG)	*matK (ycf14)*	maturase	*psbT (ycf8)*	PS II T-protein
trnS-GGA	Ser-tRNA(GGA)			*psbZ (ycf9)*	PS II core subunit
trnS-CGA	Ser-tRNA(CGA)			*petA*	b/f complex cytochrome f
trnS-UGA	Ser-tRNA(UGA)			*petG*	b/f complex subunit V
trnS-GCU	Ser-tRNA(GCU)			*petL (ycf7)*	b/f complex 3.5 kDa subunit
trnT-GGU	Thr-tRNA(GGU)			*petN (ycf6)*	b/f complex 3.2 kDa subunit
trnT-UGU	Thr-tRNA(UGU)			*ccsA (ycf5)*	c-type cytochrome synthesis
trnW-CCA	Trp-tRNA(CCA)			*atpA*	H+-ATPase subunit CF1 α
trnY-GUA	Tyr-tRNA(GUA)			*atpB*	H+-ATPase subunit CF1 β
trnV-GAC	Val-tRNA(GAC)			*atpE*	H+-ATPase subunit CF1 ε
sprA	small plastid RNA			*atpH*	H+-ATPase subunit CFO III
rps2	30S r-protein CS2			*atpI*	H+-ATPase subunit CFO IV
BIOSYNTHESIS and MISCELLANEOUS				**NADH DEHYDROGENASE SUBUNITS**	
Genes	**Products**			**Genes**	**Products**
chlB	proto chlorophyllide reduction			*ndhC*	ND3 subunit
chlL (frxC)	proto chlorophyllide reduction			*ndhD*	ND4 subunit
chlN (gidA)	proto chlorophyllide reduction			*ndhE*	ND4L subunit
cysA	transport protein			*ndhF*	ND5 subunit
cysT	transport protein			*ndhG*	ND6 subunit
accD (ycf11)	Acetyl-CoA carboxylase β subunit			*ndhH*	49 kDa protein
ycf10 (cemA, cotA)	inorganic carbon uptake			*ndhI (frxB)*	18 kDa protein

Note. Adapted from "The Genomics of Land Plant Chloroplasts: Gene Content and Alteration of Genomic Information by RNA Editing" by W. Tatsuya, T. Tsudzuki, and M. Sugiura, 2001, *Photosynthesis Research*, 70, pp.107-118.

Due to their endosymbiotic origins, chloroplasts exhibit coordinated transcription of genes within operons, functional units of DNA similar to those

found in bacteria. Operons typically contain multiple genes under the control of a single promoter, producing polycistronic mRNA that is subsequently processed into monocistrons or translated by ribosomes. Over time, the nuclear genome has incorporated many genes from the plastid, a phenomenon known as endosymbiotic gene transfer. Consequently, plastomes are greatly reduced compared to cyanobacterial genomes. Genes located in the nucleus, termed photosynthesis-associated nuclear genes, play a vital role in chloroplast development and function, alongside plastid genes. Coordinated expression of these nuclear and plastid genes is essential. Communication between the nuclear and plastid machinery occurs through anterograde signaling, while signaling from the chloroplast to the nucleus is termed retrograde signaling (Figure 13). Proteins are transported to the chloroplast via complexes such as TOC and TIC. For example, the gamma subunit of ATP synthase is delivered via these complexes. This subunit plays a crucial role in connecting the subcomplex responsible for proton translocation with the catalytic entity, thereby facilitating ATP synthesis.

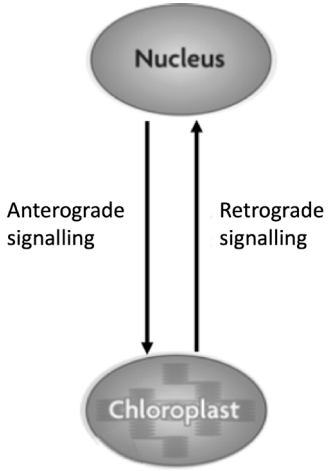


Figure 13. Plastid and nucleus communication. Adapted from: "Bilateral Communication between Plastid and The Nucleus: Plastid Protein Import and Plastidto-nucleus Retrograde Signaling" by T. Inaba, 2010, *Bioscience, Biotechnology, and Biochemistry*, 74, pp. 471-476.

Gene transcription in chloroplasts is governed by two enzymes originating from different sources: nuclear-encoded phage-type polymerase (NEP), responsible for transcribing housekeeping genes, and plastid-encoded polymerase (PEP), associated with plastid genes. The PEP holoenzyme, akin to bacterial polymerases, necessitates a specific sigma factor to recognize promoters and initiate transcription, some of which are encoded in the nuclear genome. Post-transcriptional regulation of chloroplast gene expression involves RNA processing, stabilization, splicing, and editing, facilitated by nucleus-encoded RNA-binding factors, including pentatricopeptide repeat proteins (PPR). Translation occurs through bacterial-type 70S ribosomes, progressing through initiation, elongation, and termination phases.

Chloroplasts have garnered attention from the agricultural and pharmaceutical industries due to successful chloroplast genome transformation, enabling the insertion of novel genetic information into the chloroplast database. Given their cyanobacterial origins, manipulating the chloroplast genome is more feasible than manipulating nuclear information. For instance, bacterial-like operons facilitate the introduction of multiple genes in a single step, and the absence of robust epigenetic regulation allows for the synthesis of foreign products without potential repression. However, chloroplast engineering also faces limitations, such as the lack of post-translational modifications and challenges with foreign DNA expression, which will be explored in chapter 6. Now, let's delve into the vibrant world of eukaryotic algae stemming from these endosymbiotic events.

Chapter 4

Algae of various colors

As previously discussed, several billion years ago, serial endosymbiotic events played a crucial role in the evolution of organelles. One of these events led to the emergence of primary chloroplasts, which can be traced back to the original cyanobacterial ancestor. Subsequently, organisms containing these primary chloroplasts were engulfed by heterotrophic eukaryotes, acquiring the entire photosynthetic machinery, resulting in the formation of secondary chloroplasts (Figure 14). These organisms, along with some of their descendants, collectively form what is informally referred to as algae, a polyphyletic group indicating that they do not share a recent common ancestor. While all chloroplasts are believed to have originated from cyanobacteria, the mechanisms of acquisition differ among different algal groups. For example, red and green algae, as well as glaucophytes, acquired their chloroplasts from cyanobacteria, whereas those found in diatoms and dinoflagellates originated from red algae (secondary chloroplasts). Algae encompass a wide range of organisms, including unicellular species like *Chlorella* and multicellular forms like the giant kelp. Typical features used for the primary classification of algae include the presence of an organized nucleus, the composition of pigments in the cell, the nature of stored food materials, and the length of flagella in motile cells. It's worth noting that since prokaryotes are excluded from this group, it is technically incorrect to refer to cyanobacteria as blue-green algae.

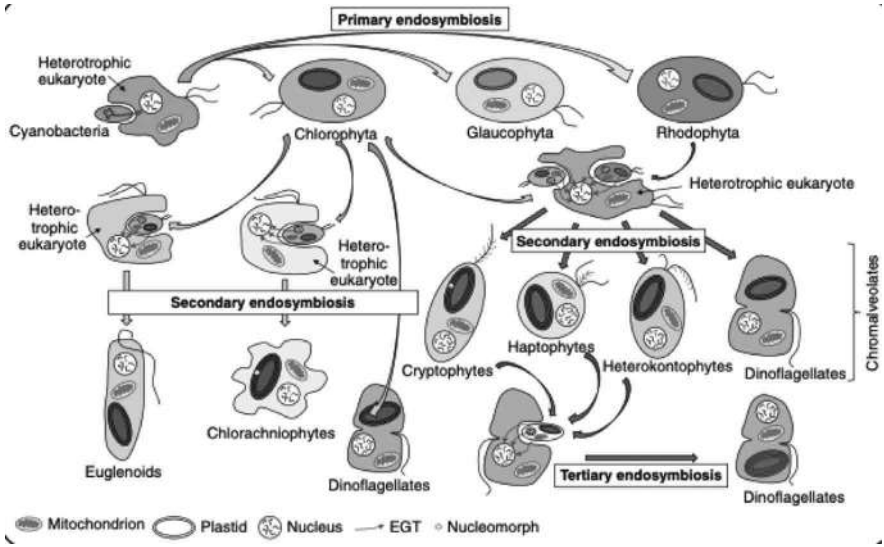


Figure 14. Microalgae evolution. Source: "Evolution of Microalgae and their Adaptations in Different Marine Ecosystems" by A. Hopes, T. Mock, 2015, *eLS*, pp. 1-9.

Among organisms harboring primary chloroplasts, three lineages can be distinguished: glaucophytes, rhodophytes (red algae), and chlorophytes (green algae) (Figure 15). Together with land plants, they constitute the Archaeplastida, sharing characteristics such as double-membrane plastids, mitochondria, cellulose-based cell walls, and starch as a storage form of food. While all synthesize chlorophyll *a*, only some produce phycobiliproteins. Glaucophytes possess cyanobacterial pigments and chloroplasts with a peptidoglycan layer (muroplasts), concentric and unstacked thylakoids, and carboxysome-like bodies. They store carbon (Floridean starch) in the cytosol and reproduce asexually by spore production, indicating their basal position within the Archaeplastida (Annex 1). Conversely, red algae produce phycobilins along with chlorophyll *a* and sometimes chlorophyll *c*, with phycoerythrin being responsible for their red coloration. They store Floridean starch outside the organelle and exhibit a wide range of forms, from unicellular to multicellular, capable of both sexual and asexual reproduction (Annex 2).

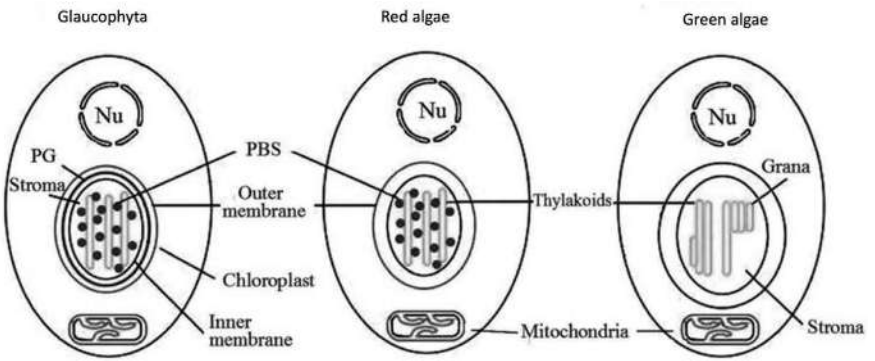


Figure 15. Cells with primary chloroplasts. Adapted from: "Phycobilisomes and Phycobiliproteins in the Pigment Apparatus of Oxygenic Photosynthetics: From Cyanobacteria to Tertiary Endosymbiosis" by I. N. Stadnichuk, V. V. Kusnetsov, 2023, *International Journal of Molecular Sciences*, 24, p. 2290.

Chlorophytes, including green algae and plants within Viridiplantae, possess chloroplasts that synthesize chlorophyll *b*, lack phycobilins, and exhibit stacked thylakoid membranes. These chloroplasts lack a peptidoglycan wall between their membranes but contain an intermembrane space. Starch storage occurs within the chloroplast, differing from glaucophytes and rhodophytes. Additionally, a microcompartment called a pyrenoid is present, aiding in carbon concentration around the RUBISCO enzyme, corresponding to cyanobacterial carboxysomes (see Figure 4). Chlorophytes have cellulose-based cell walls and reproduce both sexually and asexually. The group that includes green algae and plants is known as Viridiplantae (Annex 3). They encompass two clades: chlorophytes, primarily unicellular with some multicellular forms, and streptophytes, comprising both unicellular and multicellular organisms. Charophytes, within streptophytes, are believed to be ancestral to land plants (embryophytes). Secondary chloroplasts, derived from engulfed red or green algae by heterotrophic eukaryotes, possess more than two membranes. This secondary endosymbiotic event results in chloroplasts with three or four membranes, including those from the original plastid, the phagosomal vacuole of the eukaryote, and sometimes the membrane of the

algal cell. Vestigial eukaryotic nuclei within these chloroplasts are known as nucleomorphs (Figure 16).

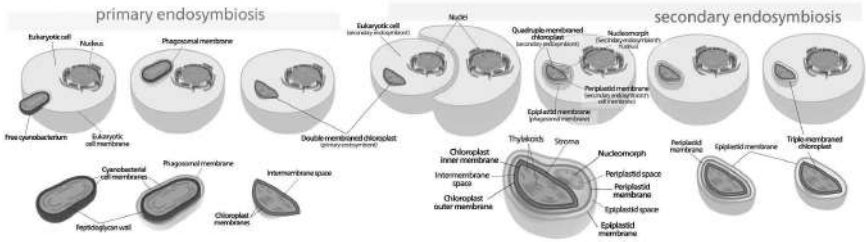


Figure 16. Endosymbiotic events that gave rise to algae. Source: File:Chloroplast endosymbiosis.svg. (2023, August 28). *Wikimedia Commons*. Retrieved September 21, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Chloroplast_endosymbiosis.svg&oldid=796574026 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).

The Euglenoids comprise flagellated unicellular eukaryotes capable of feeding via diffusion or phagocytosis, with many performing photosynthesis thanks to chloroplasts derived from green algae (Figure 14). These chloroplasts exhibit two cyanobacterial membranes and one from the host's phagosome (Annex 4), containing stacked thylakoids and a pyrenoid for carbohydrate accumulation, notably in paramylon bodies, storing glucose polymers (Figure 17). Similarly, chlorarachniophytes, marine algae, possess green algal chloroplasts with stacked thylakoids, pyrenoids, and four membranes. These organisms store carbon reserves as chrysolaminarin in the cytoplasm, alongside a nucleomorph and a periplastid compartment (PPC) representing the original green algal cytoplasm. It has been argued that the ancestor of the chlorarachniophytes originally contained a rhodoplast although it was eventually lost and replaced by a secondary chloroplast derived from green-algae (Figure 17). Dinoflagellates, single-celled marine eukaryotes, are largely photosynthetic, with chloroplasts primarily of red algal origin. However, many dinophytes have replaced their original chloroplasts with those from green algae. Notably, the mixotrophic genus *Lepidodinium* can both phagocytize and perform photosynthesis, storing starch granules in the cytoplasm, and

possessing chloroplasts with stacked thylakoids and pyrenoids enclosed by double-layered membranes, alongside a nucleomorph (Figure 17).

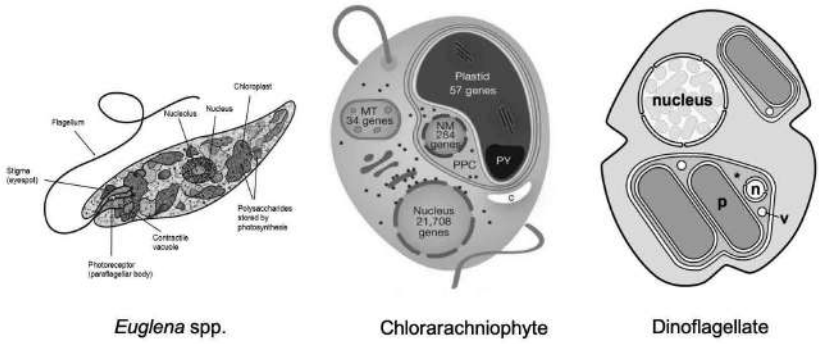


Figure 17. Cells with green-algae derived secondary chloroplasts. Adapted from: File:Euglena diagram.jpg. (2020, October 1). *Wikimedia Commons*. Retrieved September 28, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Euglena_diagram.jpg&oldid=477708577 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license https:/creativecommons.org/licenses/by-sa/4.0/deed.en); "Algal Genomes Reveal Evolutionary Mosaicism and The Fate of Nucleomorphs" by B. A. Curtis, G. Tanifuji, F. Burki, A. Gruber, M. Irimia, S. Maruyama, J. M. Archibald, 2012, *Nature*, 492, p. 59-65; "Plastid Complexity in Dinoflagellates: A picture of Gains, Losses, Replacements and Revisions" by R. F. Waller, L. Kořený, 2017, In *Advances in Botanical Research*. Publisher: Academic Press, 84, pp. 105-143.

Other algae have arisen from interactions between heterotrophic eukaryotes and red algae, resulting in the inheritance of rhodoplasts (Figure 14) (Annex 5). Cryptophytes, primarily found in freshwater but also in brackish and marine habitats, store starch in the periplastid compartment of their secondary chloroplasts. These chloroplasts possess four thylakoid membranes stacked in pairs, with pyrenoids present. They exhibit chlorophylls *a* and *c*, phycobilins, and other pigments, imparting colors ranging from brown to red to blue-green (Figure 18). Additionally, they can reproduce both sexually and asexually. Haptophytes are algae with secondary plastids featuring thylakoids stacked in threes, containing chlorophylls *a* and *c*, as well as fucoxanthin, which contributes to their brown-green hue. Pyrenoids are present, storing

chrysolaminarin in the cytoplasm, while no nucleomorph is observed (Figure 18). Heterokontophytes or stramenopiles comprise eukaryotes with plastids acquired from red algae. While most are unicellular, some, like brown algae (e.g., kelps), are multicellular. Single-celled stramenopiles are flagellated, whereas others produce flagellated cells during their life cycle, such as gametes. Photosynthetic lineages include yellow-green algae, golden algae, brown algae, and diatoms, all featuring chloroplasts arranged similarly to those of haptophytes, with pyrenoids, stacked thylakoids containing chlorophylls *a* and *c*, and fucoxanthin, contributing to their green to golden-brown coloration. These plastids are enclosed by four membranes, with the outermost connected to the endoplasmic reticulum, and sugars stored in the cytoplasm as chrysolaminarin (Figure 18).

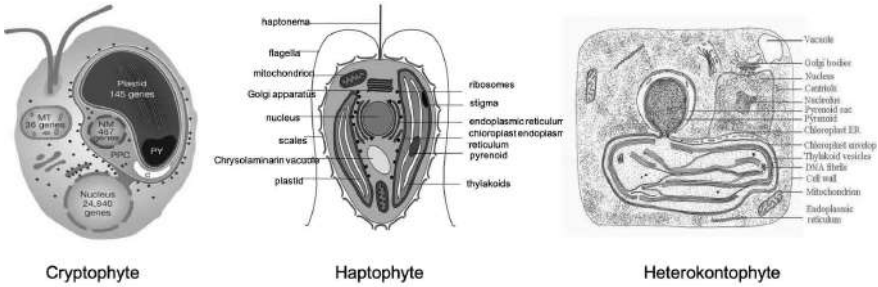


Figure 18. Cells with red-algae derived secondary chloroplasts. Adapted from: "Algal Genomes Reveal Evolutionary Mosaicism and The Fate of Nucleomorphs" by B. A. Curtis, G. Tanifuji, F. Burki, A. Gruber, M. Irimia, S. Maruyama, J. M. Archibald, 2012, *Nature*, 492, pp. 59-65; File:Haptophyta cell scheme.svg. (2020, September 17). *Wikimedia Commons*. Retrieved September 29, 2023 from https: commons.wikimedia.org/w/index.php?title=File:Haptophyta_cell_scheme.svg&oldid=463054986 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en); "*Viruses Bacteria and Thalloid Organisms*" by M. A. Khan, A. Wahid, 2006, Publisher: Higher Education Commission Islamabad, Pakistan.

As previously mentioned, dinoflagellates are unicellular eukaryotes found in marine and freshwater environments, characterized by dissimilar flagella (Figure 19). Some dinoflagellates have inherited their photosynthetic machinery from red algae (see Figure 14) (Annex 6), resulting in chloroplasts surrounded by three membranes containing chlorophyll *a*, *c2*, and β-carotene. Unique xanthophylls, including peridinin, dinoxanthin, and diadinoxanthin, contribute to their distinctive gold-brown coloration. Dinoflagellate chloroplasts, also known as peridinin-containing chloroplasts, accumulate peridinin specifically. Additionally, a pyrenoid is present, with starch accumulated outside the chloroplast. The chloroplast genome of dinoflagellates is highly reduced and often fragmented into small circles, retaining mostly photosynthesis-associated genes while others have migrated to the nuclear genome. Some dinoflagellates have lost their chloroplasts, becoming non-photosynthetic dinophytes, while others replaced them with plastids originating from algae with secondary chloroplasts, termed tertiary endosymbiosis (see Figure 14). Another notable example is the replacement of chloroplasts with green primary chloroplasts. Dinoflagellates are classified as alveolates due to the presence of flattened vesicles known as cortical alveoli beneath the plasma membrane.

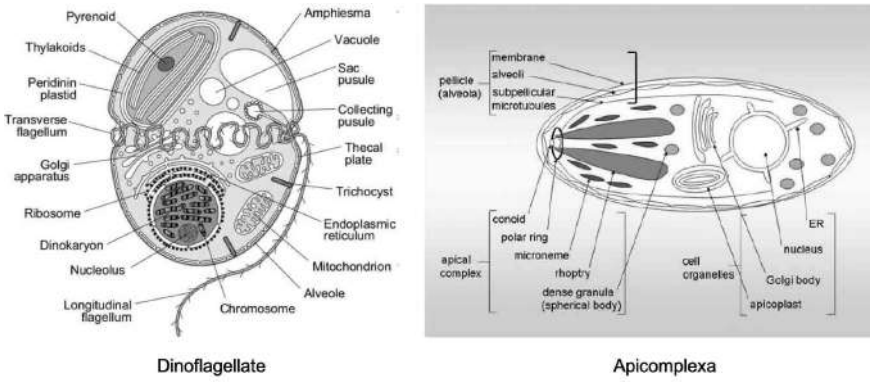


Figure 19. Alveolates. Adapted from: "Tertiary Plastid Endosymbioses in Dinoflagellates" by P. Gagat, A. Bodył, P. Mackiewicz, J. W. Stiller, 2014, In *Endosymbiosis*. Publisher: Springer. pp. 233-290; "Current and Prospective Tools for The Control of Cattle-infecting Babesia Parasites" by A. E. Rodriguez, L. Schnittger, M. L. Tomazic, M. Florin-Christensen, 2013, In *Protozoa: Biology, Classification and Role in Disease* (1st ed.). Publisher: Nova Science Publishers Inc., pp. 1-44.

In addition to dinophytes, parasitic alveolates known as Apicomplexa are identified, characterized by a non-photosynthetic organelle called the apicoplast (Figure 19) (Annex 7). Unlike true chloroplasts, apicoplasts lack thylakoids and photosynthetic pigments. These organisms store energy in the form of amylopectin, a glucose polymer, located in the cytoplasm. The apicoplast plays a crucial role in metabolic pathways such as synthesis of isoprenoid precursors, fatty acids, heme, and iron sulfur clusters. Enclosed by four membranes, the apicoplast is believed to have originated from a secondary endosymbiotic event, likely involving red algae. Importantly, it contributes to host invasion, with notorious parasites like *P. falciparum* and *T. gondii* belonging to this group, causing malaria and toxoplasmosis, respectively.

Furthermore, around 100 million years ago, a cyanobiont (see chapter 2) developed a permanent endosymbiotic relationship with an ameboid, giving rise to the photosynthetic species *Paulinella* spp (Figure 20). The resulting organelle, commonly known as a cyanelle or chromatophore, has undergone major genome reduction. Many genes were transferred to the nucleus, while others were lost, although the plastome size remains larger than that of other photosynthetic organisms. Another intriguing case involves the ciliate *Pseudoblepharisma*, which harbors two photosynthetic symbionts: green algae (*Chlorella*) and purple sulfur bacteria. However, the complexity of this symbiotic event is still not fully understood (Figure 21).

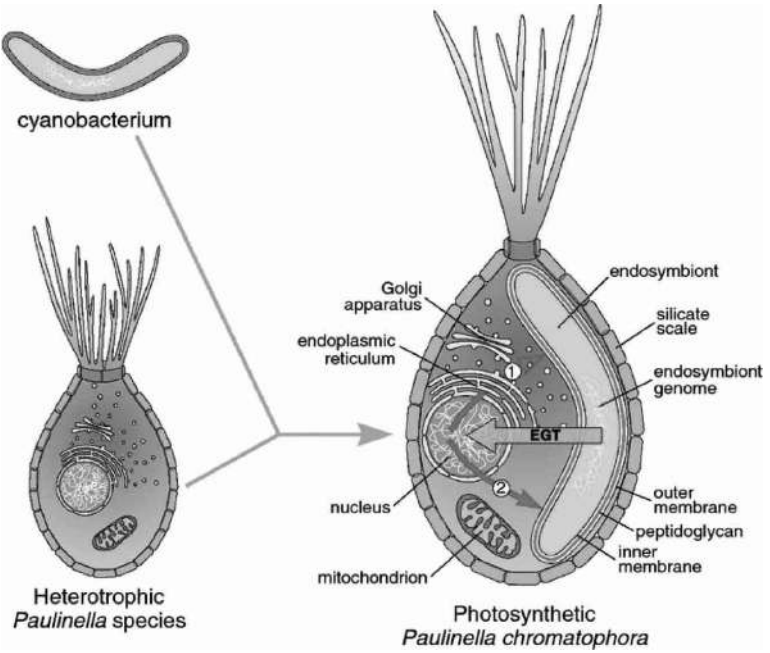


Figure 20. *Paulinella* and its plastid. Source: "Possible Import Routes of Proteins into the Cyanobacterial Endosymbionts/Plastids of *Paulinella chromatophora*" by P. Mackiewicz, A. Bodył, P. Gagat, 2012, *Theory in Biosciences*, 131, pp. 1-18.

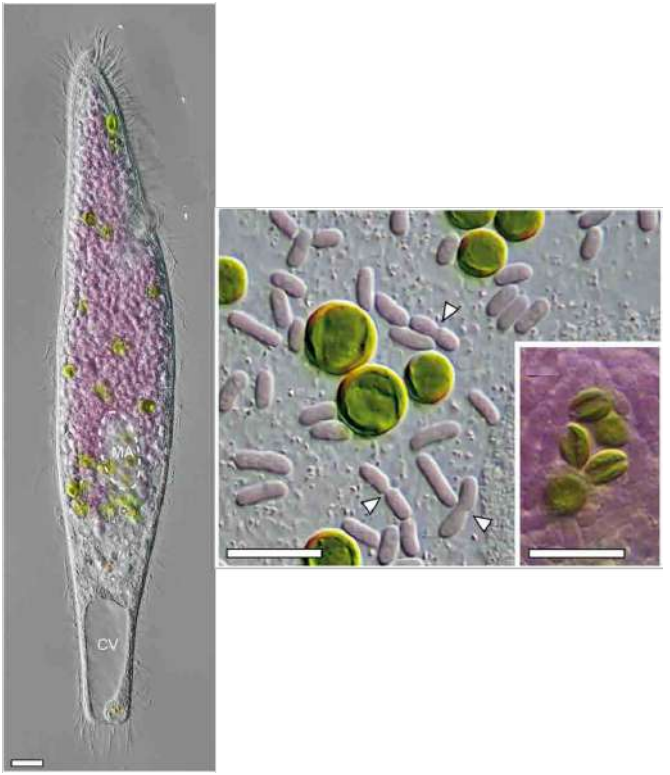


Figure 21. *Pseudoblepharisma*. Source: "A Microbial Eukaryote with a Unique Combination of Purple Bacteria and Green Algae as Endosymbionts" by S. A. Muñoz-Gómez, M. Kreutz, S. Hess, 2021, *Science Advances*, 7, eabg4102. CV, contractile vacuole; MA, elliptic macronucleus.

Certainly, humans have utilized algae for millennia, particularly in East Asia where seaweeds have been traditionally farmed for thousands of years, deeply ingrained in food culture. Today, algae farming extends beyond culinary uses to encompass bioremediation, animal feed production, pollution control, biofuel generation, and the synthesis of pharmaceutically important compounds. Genetic engineering of both chloroplast and nuclear genomes has significantly contributed to transforming algae into potential biofactories. Green algae, in particular, have been at the forefront of these advancements, with *C. reinhardtii* emerging as a pivotal organism in this field. Let's now delve into its fascinating journey.

Chapter 5

C. reinhardtii, a versatile green algal cell

C. reinhardtii, a flagellated unicellular green alga, possesses a distinctive U-shaped chloroplast housing a pyrenoid and an eyespot, facilitating phototaxis. While capable of thriving through photoautotrophic growth when exposed to light, it can also sustain growth in darkness with alternative carbon sources like acetate. Renowned as a model organism, *C. reinhardtii* has been instrumental in unraveling various biological processes. Recently, it has garnered attention for its potential in biofuel production and biopharmaceutical synthesis. Belonging to the Chlorophyta division, it shares the synthesis of chlorophyll *a* and *b*, carotenoids, and starch storage within the plastid with plants. Importantly, chlorophytes have engaged in symbiotic relationships with a range of organisms, including protozoa, sponges, fungi, and cnidarians. While predominantly found in freshwater environments, they have also been observed in marine and terrestrial habitats.

In the Chlorophyta division, the Chlorophyceae class encompasses a diverse range of organisms, spanning from unicellular to multicellular forms. These algae exhibit chloroplasts that typically adopt a discoid or cup-shaped morphology, although variations such as ribbon or spiral-like structures are also observed. Characteristically, their cell wall consists of cellulose in the inner layer and pectose in the outer layer. Reproduction in Chlorophyceae can occur through both sexual and asexual means. Asexual reproduction entails the formation of flagellated spores, while sexual reproduction involves the production of gametes. Gametes may be categorized as isogamous or

anisogamous, with isogamous gametes exhibiting similar morphologies, typically designated as plus (+) and minus (-). Conversely, anisogamous gametes differ in size and shape, with oogamy representing a specific type of anisogamy where the female gamete (ovum) is larger and immotile, while the male gamete (sperm) is smaller and motile.

Chlamydomonadales, a notable order within the Chlorophyceae class, comprises flagellated green algae, exhibiting both unicellular and colonial forms, with colonies ranging from 32 to over 500 cells (Figure 22) (Annex 8). These algae engage in the production of isogamous and anisogamous gametes, with instances of oogamy also documented. In asexual reproduction, cells divide to form new colonies, a trait believed to have evolved within the *Goniaceae* and *Volvocaceae* families (Figure 22). This order, also referred to as the Volvocales, has been extensively studied to elucidate the evolutionary mechanisms underlying multicellularity. The *Chlamydomonadaceae* family stands out, as it encompasses over 40 described genera. Species of *Chlamydomonas*, found in freshwater and moist soil environments, exhibit both sexual and asexual reproduction. While most species are phototrophic, certain species, such as *C. reinhardtii*, display facultative heterotrophy, capable of surviving in dark conditions with a source of fixed carbon, utilizing acetate through specialized molecular pathways. Unicellular and flagellated, *Chlamydomonas* cells typically exhibit oval or circular morphology and contain a cup-shaped chloroplast with associated thylakoids and a prominent pyrenoid, encased in a starch sheath at its periphery (Figure 23).

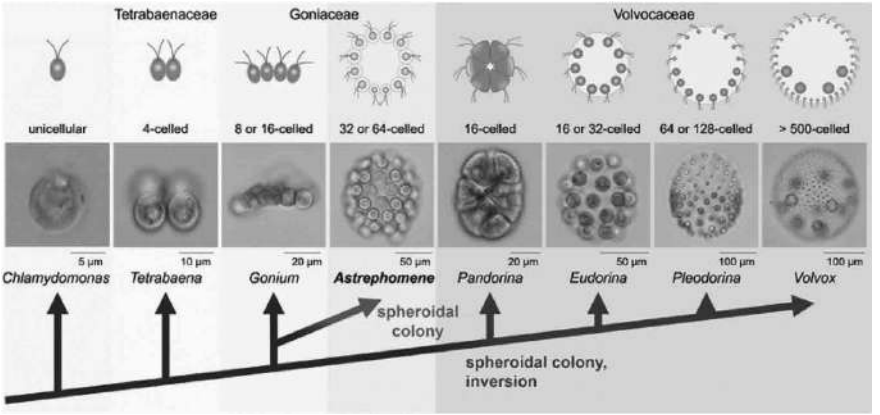


Figure 22. Volvocales and colony evolution. Source: "Alternative Evolution of a Spheroidal Colony in Volvocine Algae: Developmental Analysis of Embryogenesis in Astrephomene (Volvocales, Chlorophyta)" by S. Yamashita, Y. Arakaki, H. Kawai-Toyooka, A. Noga, M. Hirono, H. Nozaki, 2016, *BMC Evolutionary Biology*, 16, pp. 1-10.

One distinctive characteristic of these organisms is the presence of an eyespot, a photoreceptive organelle crucial for phototaxis. These structures house specific photoreceptor proteins, notably rhodopsin in the case of *Chlamydomonas*. Within *Chlamydomonas*, this photoreceptor manifests as an opsin paired with a retinal, forming channelrhodopsin—a light-gated ion channel facilitating ion influx upon light stimulation. This, in turn, alters the membrane potential, influencing flagellar movement and enabling cellular locomotion. The retinal chromophore initially exists in an all-*trans* state, transitioning to an all-*cis* state upon light absorption (at 480 nm). This transformation triggers a conformational change in the protein, opening the channel and permitting ion passage. Subsequent relaxation of the chromophore to its trans state leads to channel closure, halting ion influx. These ionotropic receptors exhibit non-specificity, allowing the transit of diverse ions such as H^+, Na^+, K^+, and Ca^{2+} (Figure 24). While eyespots are also observed in euglenoids (see chapter 3), their photoreceptors involve flavoproteins, with a flavin molecule serving as the chromophore.

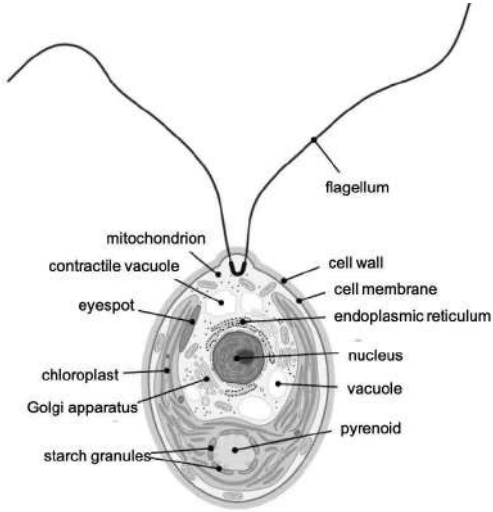


Figure 23. Morphology of *C. reinhardtii*. Adapted from: File:*Chlamydomonas reinhardtii* vector scheme.svg. (2023, April 24). *Wikimedia Commons*. Retrieved September 24, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Chlamydomonas_reinhardtii_vector_scheme.svg&oldid=753749474 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).

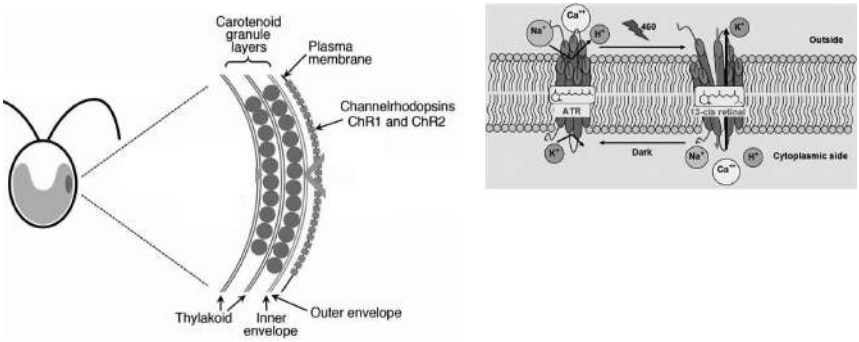


Figure 24. Structure of the eyespot. Adapted from: "Eyespot-dependent Determination of The Phototactic Sign in *Chlamydomonas reinhardtii*" by N. Ueki, T. Ide, S. Mochiji, Y. Kobayashi, R. Tokutsu, N. Ohnishi, K. I. Wakabayashi, 2016, *Proceedings of the National Academy of Sciences*, 113, pp. 5299-5304; "Development of More Light Sensitive and Red-Shifted Channelrhodopsin Variants for Optogenetic Vision Restoration" by T. H. Ganjawala, 2019, *Doctoral Dissertation*, Wayne State University.

The name "*Chlamydomonas*" derives from the proteinaceous cell wall enveloping the cell, resembling a cloak (where "chlamys" means cloak and "monas" denotes a unit or solitary entity), and "*reinhardtii*" honors the botanist Ludwig Reinhardt, who first described it. *C. reinhardtii* exhibits versatile feeding habits, capable of autotrophic, heterotrophic, or mixotrophic nutrition modes. Asexually, it reproduces via haploid vegetative cells containing 17 chromosomes, while sexual reproduction involves isogamous gametes classified as plus or minus, often induced by nitrogen depletion or blue light exposure. Its nuclear, mitochondrial, and chloroplast genomes have been sequenced, facilitating its role as a model organism. The rapid growth of haploid cells enables immediate expression of mutant phenotypes, while the ability to utilize acetate aids in studying light-sensitive mutants and electron transport chain dynamics. Importantly, *C. reinhardtii* played a pivotal role in identifying key PSII proteins (D1, D2) and discovering channelrhodopsins, advancing optogenetics. Current research delves into elucidating the cell cycle, basal body development, pyrenoid structure, light acclimation, ecological interactions, and genomic manipulation for biotechnological applications (Figure 25).

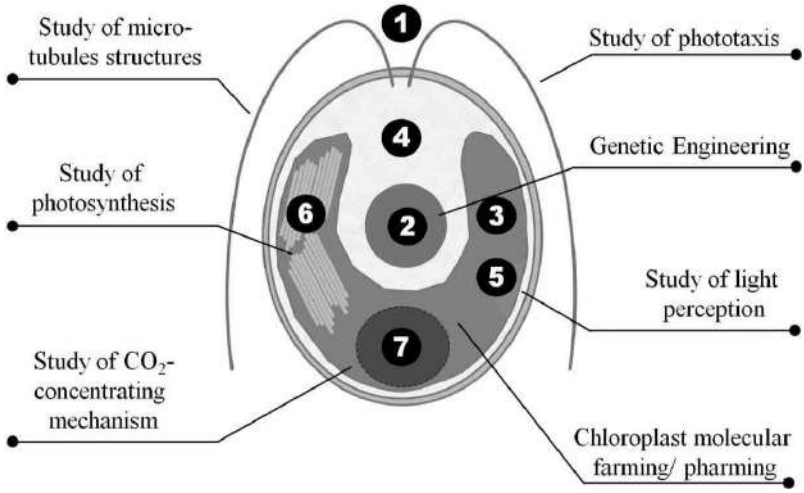


Figure 25. *C. reinhardtii* and biological phenomena. Source: "Achievements and Challenges of Genetic Engineering of the Model Green Alga *Chlamydomonas reinhardtii*" by N. T. Tran, R. Kaldenhoff, 2020, *Algal Research*, 50, 101986.

In addition to the previously mentioned morphological traits, internal transcribed spacers serve as crucial tools for classification. While phenotypic characteristics aid in distinguishing among genera, they often fail to provide insights at the species level due to their similar appearances. Internal transcribed spacers, located between rRNA genes, offer DNA sequences for comparing and assessing taxonomic relationships among organisms. *Chlamydomonas* spp. have been documented in temperate, subtropical, and tropical regions, yet confirmed instances of *C. reinhardtii* have predominantly been isolated from temperate habitats. This species thrives in nutrient-rich soils, particularly in the upper millimeters where ample light is available. It is suggested that these microorganisms contribute to primary production and may act as pioneer species, colonizing barren environments and subsequently providing nutrients for the growth of other species. *C. reinhardtii* is also found in aquatic environments, primarily inhabiting the pelagic zone, which denotes the zone in a lake or ocean water column that is not near the bottom.

In such habitats, motility confers a competitive advantage, especially in stratified lakes. The microalga's ability to swim facilitates optimal light exposure and nutrient acquisition by descending the water column. Growth limitations include phosphorus and nitrogen availability, light availability, and predation pressure. All laboratory strains originate from a single zygote isolated from a Massachusetts potato field. Under laboratory conditions, one mutation emerges per 30 generations, leading to specific traits in these strains, such as a preference for ammonia over nitrate utilization, rendering their survival in natural habitats challenging.

When faced with adverse conditions, haploid vegetative cells of *C. reinhardtii* have a remarkable adaptive strategy: they transition into diploid cells or zygotes. In laboratory environments, cues like nitrogen deprivation and reduced light trigger signals indicating deteriorating conditions, prompting the initiation of gametogenesis. Haploid cells then fuse to form zygotes, which give rise to dormant zygospores. Conversely, when nitrogen and light become available, zygotes undergo meiosis, generating haploid cells that resume vegetative growth (Figure 26). Zygospores exhibit remarkable resilience, remaining viable in soil for years and tolerating freezing and desiccation, attributes associated with the robust multilayered cell wall composed of a durable lipid polymer. Sexual reproduction enhances the rate of adaptation to changing environmental conditions by fostering genetic diversity. Upon zygote formation, transcription factors instigate zygote development, with some genes being expressed within minutes and others over subsequent hours or days. Notably, the gene responsible for encoding polyketide synthase becomes active two days post-fertilization, playing a crucial role in the transition from zygote to zygospore and in the synthesis of cell wall lipid polymers.

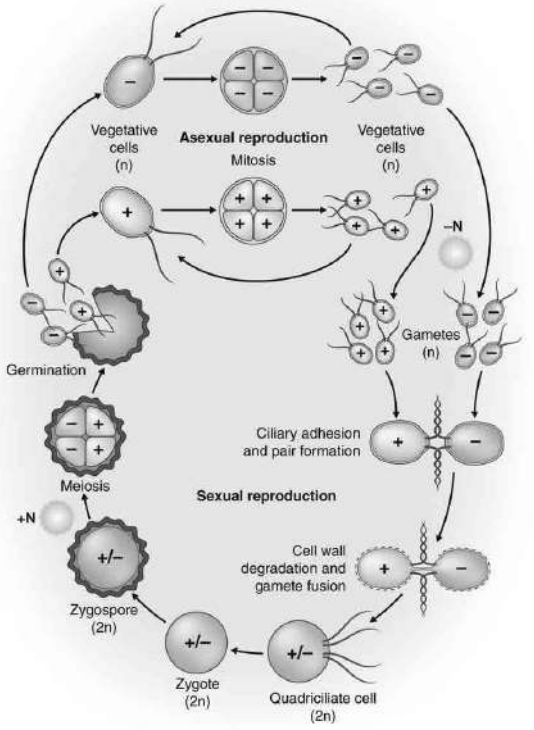


Figure 26. *C. reinhardtii* life cycle. Source: "From Molecular Manipulation of Domesticated *Chlamydomonas reinhardtii* to Survival in Nature" by S. Sasso, H. Stibor, M. Mittag, A. R. Grossman, 2018, *Elife*, 7, e39233.

The motility of these cells confers a significant advantage, especially in nocturnal or subterranean environments, aiding in their navigation and movement to locate favorable conditions. Vegetative cells exhibit attraction to compounds like ammonium, nitrite, and nitrate. In anaerobic conditions, they rely on glycolysis for energy production, supplemented by fermentation. These cells demonstrate the capacity for both photoautotrophic and heterotrophic growth. Heterotrophic metabolism is sustained by the breakdown of 2-carbon molecules like acetate, with carbon from these sources entering the citric acid cycle to fuel heterotrophy.

Glucose synthesis is achieved through gluconeogenesis, wherein non-hexose precursors are converted into glucose. This metabolic versatility appears to be an evolutionary adaptation to habitats characterized by limited light availability and low oxygen levels.

C. reinhardtii cells possess three distinct genomes: one housed within the nucleus, and the other two residing in the mitochondria and chloroplasts, respectively. The mitochondrial genome, a linear molecule spanning 15.8 kb, encodes eight proteins along with tRNA and rRNA essential for respiration, with the organelle relying on tRNA and protein import from the cytosol. While transformation of the mitochondrial genome has been achieved, it occurs with limited efficiency. The nuclear genome spans 111 Mb across 17 chromosomes, comprising approximately 17,741 loci encoding 19,525 proteins (including variants due to alternative splicing). Most genes feature introns and typically consist of seven exons, while transcripts exhibit relatively long untranslated regions (UTRs). Lastly, the chloroplast genome, a 250-kb molecule with a high repeat content predominantly in intergenic regions, contains nearly one hundred genes, encompassing those coding for tRNA, rRNA, ribosomal, and photosynthesis-related proteins. Detailed exploration of plastomic gene expression and genome engineering constitutes the focus of the upcoming chapters.

Chapter 6

Chloroplast genome of *C. reinhardtii*

The chloroplast genome contains crucial genes responsible for the photosynthetic apparatus and the elements required for their expression and assembly. Alongside genes related to photosynthesis, it also includes those for polymerases and translation machinery. Nevertheless, the expression of these genes relies on diverse factors encoded in the nuclear genome and imported from the cytoplasm.

Before exploring the intricacies of the plastome, it is beneficial to review some fundamental molecular biology concepts. At its core, a gene is a sequence of nucleotides that encodes a specific function. This DNA segment is transcribed into RNA, which, in certain cases, is further translated into proteins. Genes are organized within chromosomes, each occupying a distinct locus or position. (Figure 27). The genome, or genotype, represents the entire collection of genes and genetic instructions that define an organism, while the phenotype is the observable outcome of those genetic traits. DNA is a double-stranded molecule composed of nucleotides, featuring a sugar-phosphate backbone and inward-facing nitrogenous bases. The ends of the DNA strand are labeled as 5' (prime) and 3', denoting the carbon atoms on the deoxyribose ring associated with phosphate (5') and hydroxyl (3') groups. Nucleotides pair specifically within the DNA molecule, with adenine pairing with thymine and guanine pairing with cytosine (Figure 28).

During cell division, both in prokaryotes and eukaryotes, the entire genome is copied in a process termed DNA replication. This intricate process

involves a coordinated effort of various enzymes forming the replisome complex (Figure 29). Initially, DNA is unwound into single strands at a designated site known as the origin of replication. Specialized proteins bind to this site and separate the DNA strands. A key enzyme called helicase then intervenes, disrupting hydrogen bonds and unwinding the double helix. As helicase progresses along the DNA, topological changes occur, resulting in supercoiling. Topoisomerases are enzymes that alleviate this coiling by cutting the DNA strands, allowing them to rotate and relieve the supercoil. Eventually, the DNA strands are rejoined. To prevent inappropriate pairing events, single-strand binding proteins interact with exposed bases, ensuring stability during replication.

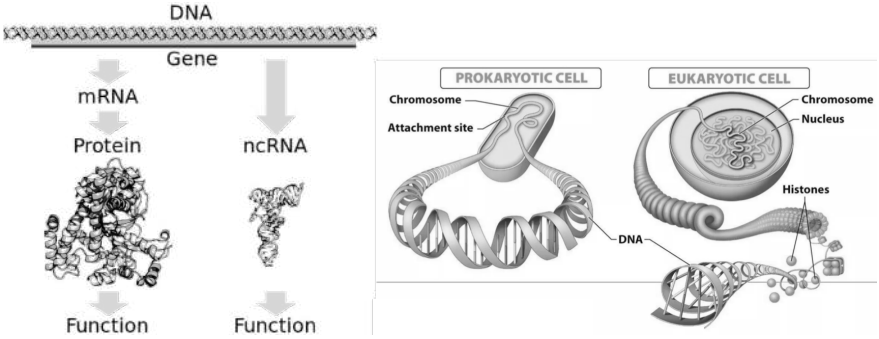


Figure 27. Genes and chromosomes. Adapted from: File:DNA to protein or ncRNA. svg. (2021, March 11). *Wikimedia Commons*. Retrieved September 28, 2023 from https://commons.wikimedia.org/w/index.php?title=File:DNA_to_protein_or_ncRNA.svg&oldid=541344309 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en); "*Prokaryotic and Eukaryotic chromosomes*". (n.d.). IB BIOLOGY. Retrieved September 28, 2023 from https://www.slideshare.net/smullen57/ib-biology-32-chromosomes.

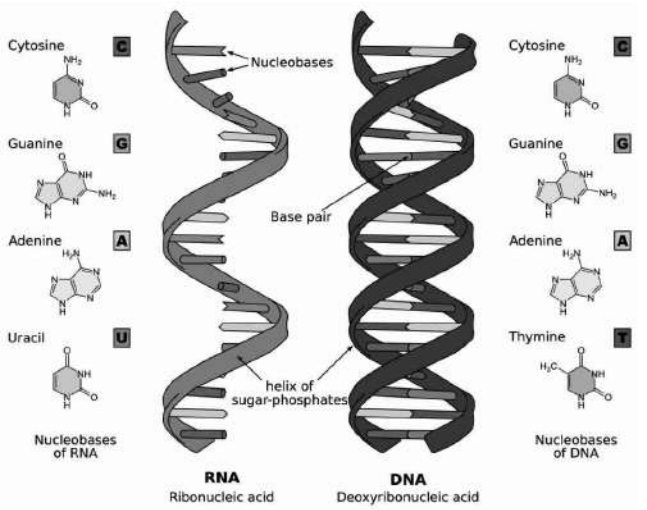


Figure 28. DNA and RNA structure. Source: "*DNA Replication*". (n.d.). TEACH ME PHYSIOLOGY. Retrieved October 2, 2023 from https://teachmephysiology.com/biochemistry/cell-growth-death/dna-replication/.

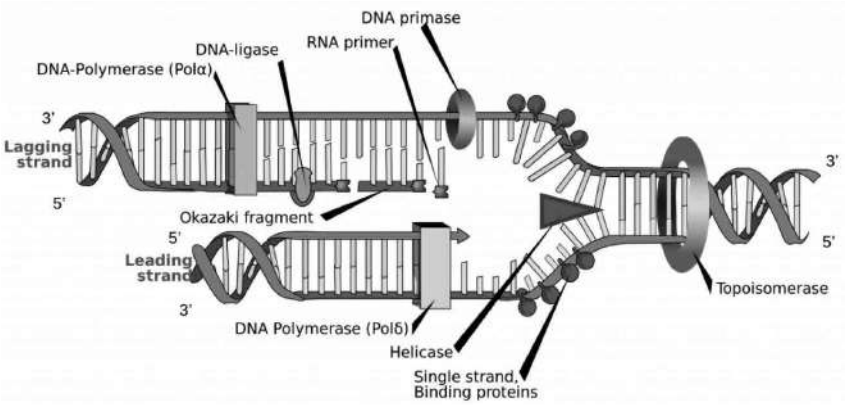


Figure 29. DNA replication. Source: "*DNA Replication*". (n.d.). TEACH ME PHYSIOLOGY. Retrieved October 2, 2023 from https://teachmephysiology.com/biochemistry/cell-growth-death/dna-replication/.

DNA polymerases play a crucial role in synthesizing new DNA strands, necessitating a starting point for their action. Initially, a short RNA primer is synthesized by an RNA polymerase, providing a specific location for

DNA polymerase to commence nucleotide addition. This enzyme catalyzes the formation of phosphodiester bonds between the 3' OH terminus of the growing strand and free nucleotides' phosphorus atoms, facilitating synthesis in a 5' to 3' direction. The leading strand is synthesized continuously in the direction of the replication fork and requires only one primer. Conversely, the lagging strand is synthesized in fragments, known as Okazaki fragments, each necessitating its own primer. Subsequently, RNA molecules are replaced with DNA, and DNA ligase seals the gaps in the sugar backbone by forming bonds between the fragments. Ultimately, this process results in the generation of sequences that faithfully replicate the original DNA strands (Figure 29).

The process of gene expression involves transcription, where the genetic message encoded in DNA is transcribed into RNA (Figure 30, left-hand side). RNA polymerase, aided by transcription factors, binds to a specific DNA sequence called the promoter and synthesizes an RNA molecule using one DNA strand as a template. The other DNA strand, known as the coding strand, has a sequence complementary to the RNA transcript. Transcription occurs in three main stages: initiation, elongation, and termination. During initiation, the transcription machinery assembles at the promoter region to initiate RNA synthesis. In the elongation phase, RNA polymerase moves along the DNA template, adding RNA nucleotides in a 5' to 3' direction to elongate the RNA molecule. Finally, termination sequences signal the end of transcription, and the RNA polymerase detaches from the DNA, allowing the DNA to return to its original state. In prokaryotes, transcription takes place in the cytoplasm, and transcripts are immediately available for translation. In contrast, in eukaryotes, transcription occurs in the nucleus. The primary RNA transcript undergoes processing, including splicing and addition of a 5' cap and a poly-A tail, before being transported to the cytoplasm for translation into proteins (Figure 30, right-hand side).

The primary transcript resulting from transcription contains both exons, which are coding sequences arranged in codons, and introns, which are non-coding RNA segments that must be removed (see below). This removal process, known as splicing, is facilitated by the spliceosome, a ribonucleoprotein

complex that cleaves out the introns and ligates the flanking exons together. Alternative splicing is a crucial mechanism where different combinations of exons can be included or excluded, leading to the production of diverse mRNA strands and ultimately different proteins (Figure 31). In addition to splicing, the primary transcript undergoes modifications at both ends. At the 5' end, a methylated guanosine cap is added to the first nucleotide, which enhances nuclear export, protects the mRNA from degradation, and promotes ribosome binding. At the 3' end, polyadenylation occurs, where approximately 250 adenine bases are added by a polyadenylate polymerase. This poly(A) tail serves as a buffer against exonucleases and contributes to mRNA stabilization. Together, these modifications ensure the efficient processing and stability of the mRNA molecule (Figure 30, right-hand side).

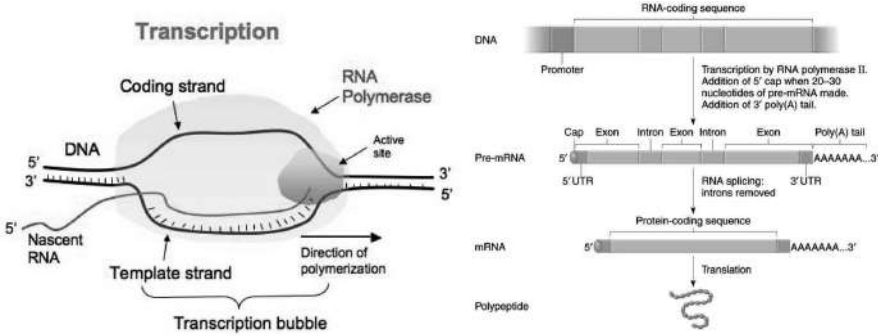


Figure 30. Transcription and RNA processing. Adapted from: Karki, G. (2017, June 21). "*Transcription in Prokaryotes*". ONLINE BIOLOGY NOTES. Retrieved October 5, 2023 from https://www.onlinebiologynotes.com/transcription-inprokaryotes/; Carr, S. M. (2014). "*Post-transcriptional Processing of RNA in Eukaryotes*". NEWFOUNDLAND AND LABRADOR'S UNIVERSITY. Retrieved October 5, 2023 from https://www.mun.ca/biology/scarr/iGen3_05-12.html.

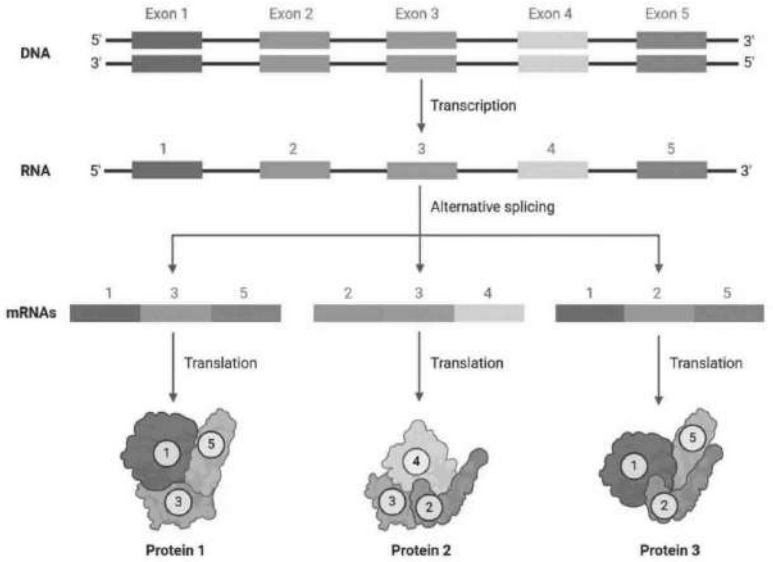
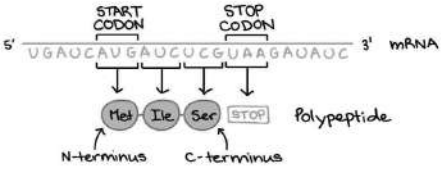


Figure 31. Alternative splicing. Source: Saptoka, A. (May 8, 2022). "*RNA Splicing-Definition, Process, Mechanism, Types, Errors, Uses*". MICROBE NOTES. Retrieved October 6, 2023 from https://microbenotes.com/rna-splicing/.

After transcription and processing, the mRNA molecule is ready for translation by the ribosome, where it is decoded into a string of amino acids to form a protein. Each group of three nucleotides in the mRNA sequence, known as a codon, corresponds to a specific amino acid. With four different nucleotides available, there are 64 possible codon combinations (4^3 = 64). However, since there are only 20 amino acids used in protein synthesis, some codons code for the same amino acid, while three codons serve as stop signals to halt translation. The start codon, AUG, initiates protein synthesis and codes for the amino acid methionine (Figure 32). During translation, transfer RNA (tRNA) molecules play a crucial role by carrying specific amino acids to the ribosome. Each tRNA molecule has an anticodon that matches the codon on the mRNA, ensuring accurate pairing and incorporation of the correct amino acid into the growing polypeptide chain (Figure 33). The ribosome, composed of two subunits containing RNA and proteins, facilitates the translation process. The small subunit contains an RNA molecule and several proteins, while

the large subunit consists of three RNA molecules and additional proteins. Together, these subunits coordinate the binding of mRNA and tRNA, catalyzing the formation of peptide bonds between amino acids to synthesize proteins. During translation, both the small and large ribosomal subunits interact with transfer RNA (tRNA) molecules through three distinct sites: the aminoacyl site (A), the peptidyl site (P), and the exit site (E). At the A site, tRNA molecules dock, carrying specific amino acids. The peptidyl site (P) links the growing peptide chain to the charged amino acid, while the exit site (E) facilitates the dissociation of deacylated tRNA from the complex (Figure 33). Translation continues until the ribosome encounters a stop codon on the mRNA. At this point, a release factor interacts with the ribosome, triggering the disassembly of the entire complex. These fundamental processes summarize the key events involved in gene expression. Notably, significant differences exist between prokaryotic and eukaryotic transcription and translation processes (Annex 9).



Second letter

First letter	U	C	A	G	Third letter
U	UUU, UUC: Phe UUA, UUG: Leu	UCU, UCC, UCA, UCG: Ser	UAU, UAC: Tyr **UAA Stop** **UAG Stop**	UGU, UGC: Cys **UGA Stop** UGG Trp	U C A G
C	CUU, CUC, CUA, CUG: Leu	CCU, CCC, CCA, CCG: Pro	CAU, CAC: His CAA, CAG: Gln	CGU, CGC, CGA, CGG: Arg	U C A G
A	AUU, AUC, AUA: Ile AUG: Met	ACU, ACC, ACA, ACG: Thr	AAU, AAC: Asn AAA, AAG: Lys	AGU, AGC: Ser AGA, AGG: Arg	U C A G
G	GUU, GUC, GUA, GUG: Val	GCU, GCC, GCA, GCG: Ala	GAU, GAC: Asp GAA, GAG: Glu	GGU, GGC, GGA, GGG: Gly	U C A G

Figure 32. Codons and amino acids. Source: "*The Genetic Code*". (n.d.). KHAN ACADEMY. Retrieved October 6, 2023 from https://www.khanacademy.org/science/ ap-biology/gene-expression-and-regulation/translation/a/the-genetic-code-discoveryand-properties.

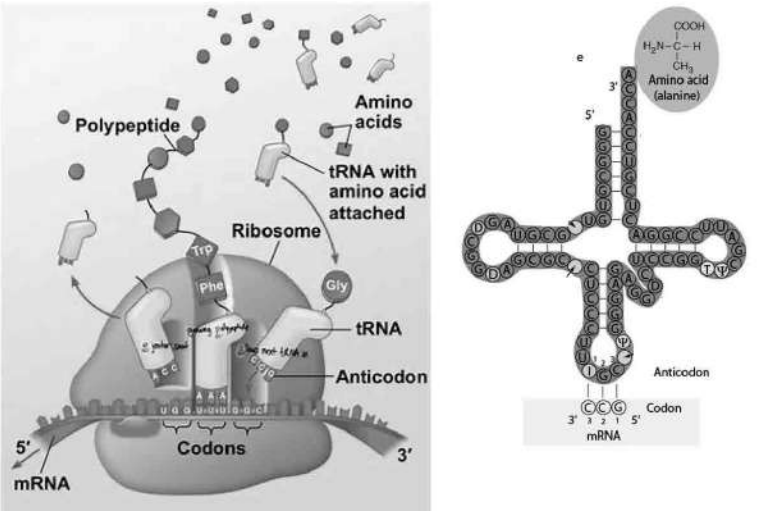


Figure 33. Translation. Adapted from: "*DNA Translation in Biology*". (n.d.). CHEMISTRY TALK. Retrieved October 6, 2023 from https://chemistrytalk.org/dna-translation-in-biology/; "*Transfer RNA*". (n.d.). HYPERPHYSICS. Retrieved October 6, 2023 from http://hyperphysics.phy-astr.gsu.edu/hbase/Organic/trna.html.

Now that we have explored the process of gene expression, let's now return to the focus of this chapter: the chloroplast genome. This circular molecule spans approximately 200 kb and encompasses 99 genes with known functions (Figure 34). Remarkably, there is a prevalence of A + T content, constituting around 65% of the genome. The chloroplast genome can be dissected into four distinct regions: two single copies and two inverted repeats. The single-copy regions vary in size, comprising small and large versions, and host the majority of protein-coding genes alongside certain tRNA genes. Conversely, the inverted repeats maintain consistent sizes and encompass sequences pertinent to photosynthesis, in addition to tRNA and rRNA genes. Some genes are organized into clusters, particularly those linked to PSII and ribosomal proteins. Of the total gene count, 64 are responsible for encoding proteins, 30 for tRNAs, 6 for rRNAs, and the remainder for proteins with diverse functions (Figure 34). These protein-coding genes can be categorized into three main groups: firstly, those involved in the photosynthetic apparatus, such as the *psb* (PSII) and *psa* (PSI) genes, as well as the *pet* (cytochrome) and *atp* (ATP

synthase) genes. Secondly, genes associated with the expression machinery, including polymerases and ribosomal proteins. The last group encompasses sequences associated with various functions, such as maintaining photosystem stability and facilitating cytochrome *b6f* formation. These genes exhibit distinctive characteristics. For example, both type I and type II introns have been identified, with the former found in the 23S rRNA and *psbA* genes, and the latter in the *psaA* gene. Additionally, four origins of replication have been pinpointed, along with the identification of two proteins possessing DNA polymerase activity.

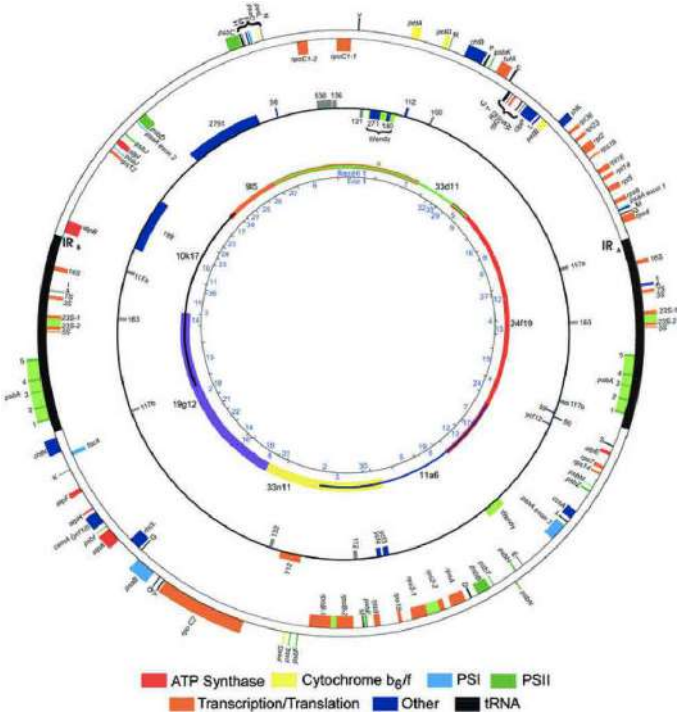


Figure 34. The chloroplast genomes of *C. reinhardtii*. Source: "The *Chlamydomonas reinhardtii* Plastid Chromosome: Islands of Genes in a Sea of Repeats" by J. E. Maul, J. W. Lilly, L. Cui, C. W. DePamphilis, W. Miller, E. H. Harris, D. B. Stern, 2002, *The Plant Cell*, 14, pp. 2659-2679.

Chloroplast genes exhibit diverse transcriptional patterns, with some transcribed individually (monocistrons), while others are part of operons,

generating primary transcripts that necessitate further processing. Co-transcription is prevalent in chloroplasts, with around 70% of transcripts being polycistronic. The transcription machinery resembles that of prokaryotes, featuring characteristic sigma^{70}-type promoters containing -35 (TTGACA) and -10 (TATAAT) sites. Variants lacking the -35 site and an extended -10 element (TATAATATA) have also been identified. Additionally, a distinct type of promoter associated with tRNAs has been recognized but not fully characterized. Chloroplasts employ a unique DNA-dependent RNA polymerase known as PEP (plastid-encoded polymerase), differing significantly from the transcriptional machinery observed in plant chloroplasts, which typically contain both a PEP and a NEP (nucleus-encoded polymerase). Stem-loop structures present in plastid mRNAs serve to pause the polymerase enzyme and facilitate its release; the formation of these structures is facilitated by inverted repeats commonly found in the 3' UTRs. Indeed, some chloroplast genes harbor introns within their sequences. As mentioned earlier, introns present in chloroplast genes need to be removed through a process called splicing. While some genes like *psbA* and *rrn* have introns capable of self-splicing, others like *psaA* require various factors for excision. These factors, encoded in the nucleus, are subsequently imported into the chloroplast through the TOC and TIC complexes (as discussed in Chapter 3). The exons of *psaA* are dispersed throughout the chloroplast genome, and only after transcription are they assembled into a primary transcript that includes two introns and a long non-coding RNA called *tscA*. Subsequently, trans-splicing factors remove the introns, allowing the exons to join together and form the mature molecule (Figure 35).

In addition to splicing, several other peculiarities have been observed in chloroplast gene expression. Transcript processing occurs not only at the 5' and 3' ends but also internally, particularly in polycistronic molecules. This internal processing involves factors imported from the cytoplasm. RNA degradation plays a critical role in removing damaged molecules, recycling nucleotides, and regulating gene expression. Both exo- and endonucleases are involved in this process. The 3' untranslated regions (UTRs) are essential for

RNA degradation, as they contain inverted repeats that facilitate the formation of stem-loop structures. For example, the *atpB* gene includes such sequences in its 3' UTRs. Similarly, the 5' UTR is crucial for promoting RNA stability. In the *psbD* gene, this region contains sequences that enhance the abundance of mature RNAs. Factors imported from the nucleus interact with these regions, facilitating transcript processing. The availability of light and phosphorus also plays a crucial role in regulating transcript levels.

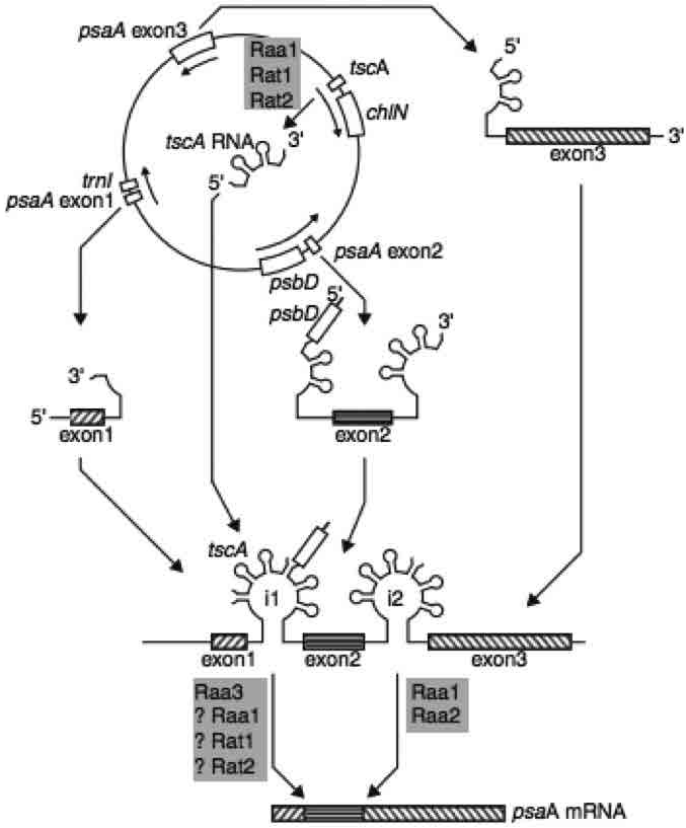


Figure 35. Splicing in the organelle. Adapted from: "Chloroplast RNA Metabolism" by D. B. Stern, M. Goldschmidt-Clermont, M. R. Hanson, 2010, *Annual Review of Plant Biology*, 61, pp. 125-155.

Despite its cyanobacterial origins, the chloroplast has not retained all of its prokaryotic characteristics but has acquired novel features. Chloroplast ribosomes, for example, contain not only bacterial-like proteins but also a set of proteins specific to the organelle known as plastid-specific ribosomal proteins (PSRP). These PSRPs are important for regulating mRNA translation within the chloroplast. The 70S ribosomes found in chloroplasts have counterparts for some proteins found in the ribosomal subunits of *E. coli* and plant ribosomes. Some of these proteins are similar to those found in spinach and are encoded in the nuclear genome. Their products are later imported into the chloroplast via transit peptides. In addition to protein composition, the organization of ribosomal genes in chloroplasts is noteworthy. They are arranged in an operon fashion, resulting in the production of polycistronic molecules that require processing to yield mature tRNAs and rRNAs. Both the small and large subunits of the chloroplast ribosome play essential roles in translation. The small subunit is involved in codon/anticodon pairing, directly impacting translational efficiency, while the large subunit is associated with productivity. Various antibiotics target chloroplast ribosomes. For instance, streptomycin and spectinomycin bind to the small subunit, whereas erythromycin and chloramphenicol bind to the large subunit. Resistance to these antibiotics can arise through different mechanisms, such as modification of the targeted sequence, alteration of ribosomal proteins, and protein-mediated inactivation.

In chloroplasts, translation factors responsible for initiation, elongation, and termination are GTPases, similar to those found in *E. coli*. However, these factors are encoded in the nuclear genome rather than in the chloroplast genome. They interact with specific sequences in the 5' untranslated regions (UTRs) of mRNAs to regulate translation. The translation of certain chloroplast proteins, such as the D1 protein that forms part of the reaction center of Photosystem II (PSII), is modulated by light. During photosynthesis, changes in the redox state of the cell trigger the recruitment of proteins that facilitate the loading of *psbA* mRNA onto the ribosome, allowing translation of the D1 protein to proceed. Similarly, the transcript of the *psbD* gene is regulated by a protein called Nac2. Nac2 contributes to the initiation of translation and protects the

mRNA from degradation by exonucleases. Overall, the functional metabolism of the chloroplast relies on the coordinated action of products encoded by both the nuclear and plastid genomes. This cooperation ensures the proper expression of genes essential for chloroplast function and photosynthesis.

The chloroplast's relatively simple yet highly efficient system makes it an attractive platform for recombinant protein production. Additionally, the organelle hosts numerous metabolic pathways that can be redirected for the synthesis of valuable metabolites. Utilizing the plastome for this purpose offers several advantages. For example, gene targeting through homologous recombination enables precise integration, thereby avoiding random integration events. Moreover, the chloroplast allows for the attainment of relatively high levels of protein expression, with proper folding and correct disulfide-bond formation being observed. Engineered strains can be endowed with the capacity to produce high-value products, leveraging light energy to drive the synthesis of important molecules. In the following chapter, we discuss the intricacies of transplastomic engineering in *C. reinhardtii*.

Chapter 7

Transplastomic engineering of *C. reinhardtii*

As mentioned earlier, the chloroplast possesses a polyploid genome inherited from its cyanobacterial progenitor. The majority of genes housed within the plastome are linked to photosynthesis, as well as transcription and translation processes. This genomic setup has preserved certain bacterial traits, including the presence of a eubacterial-like RNA polymerase, a 70S ribosome, and co-transcribed units. However, some genes exhibit introns, and gene expression regulation primarily occurs post-transcriptionally, differing from bacteria where regulation typically is observed at the transcriptional level. This regulatory mechanism is orchestrated by *cis* factors present on mRNA molecules and *trans* factors imported from the cytoplasm.

The transformation of the chloroplast genome was initially accomplished in the late 1980s using *C. reinhardtii*, marking a significant milestone in biotechnology. Since then, the techniques for engineering this microalga have been well-refined. Furthermore, successful transformation of chloroplasts has been achieved in various other microalgae species, including *Dunaliella tertiolecta*, *Haematococcus pluvialis*, *Cyanidioschyzon merolae*, and *Phaeodactylum tricornutum*. Additionally, plants such as tobacco, tomato, and potato have served as hosts for transplastomic engineering. The ability to cultivate microalgae in closed systems and under controlled conditions, such as bioreactors, coupled with their generally recognized safety profile, renders them an attractive platform for expressing valuable products. Importantly, the inherent safety of microalgae like *C. reinhardtii* raises the possibility of utilizing

their cell lysates as an alternative to purified proteins, further enhancing their utility in biotechnological applications. Furthermore, dried cells offer a natural encapsulation system, enabling the expression of proteins that typically require cold-chain transportation. Various bioproducts such as antimicrobials, vaccines, enzymes, and hormones have been successfully synthesized from the plastome of *C. reinhardtii*. However, the introduction of foreign DNA into the chloroplast is a prerequisite for recombination to occur. This can be achieved through three primary methods: (i) Biolistics, where DNA-coated gold particles are propelled into a lawn of cells, (ii) electroporation, which involves the application of an electrical current to create transient holes in the membrane for DNA passage, and (iii) agitation with glass beads, where a cell wall-less mutant is vortexed along with the microalgae culture and foreign DNA, with the glass beads facilitating the creation of transient holes in the membranes for vector entry into the chloroplast and subsequent recombination.

Integration of foreign DNA into the chloroplast genome occurs through homologous recombination facilitated by sequences present in both the vector and the chromosome. Due to the polyploid nature of the genome, only a subset of copies accepts the foreign cassette after transformation, resulting in a mixture of genomes, a phenomenon referred to as heteroplasmy. The objective is to promote the replication of transformed genomes, typically achieved through the application of selective pressure. Following multiple rounds of selection, homoplasmy is achieved, wherein all copies of the genome harbor the transgene (Figure 36). Successful expression relies heavily on vector design, necessitating careful consideration of various parameters. Primarily, *cis* factors, crucial for proper transcription and translation, interact with *trans* factors; therefore, expression sequences are typically derived from endogenous genes (Figure 37) (Table 2). For instance, promoters, 5' and 3' UTRs essential for expression, transcript stability, or translation initiation are often sourced from genes associated with the photosynthetic machinery, such as *psaA*, *petB*, or *atpA*. Several factors contribute to successful vector design. Firstly, utilizing a robust promoter associated with the *rrn* gene of the 26S subunit has proven effective. Secondly, considering the bias towards A-T codons in the genome

due to its adenine and thymine richness, transgene sequences often require codon optimization to ensure proper translation. Thirdly, accurate targeting of the transgene is paramount to prevent potential genetic disruptions; employing appropriate sequences for homologous recombination facilitates cassette integration into neutral sites. Lastly, selecting an appropriate selection method is crucial given the low probability of DNA delivery. However, certain considerations must be envisaged, including the possibility of cells evading selective pressure (false-positives), and the potential risks associated with using markers based on antibiotic resistance genes (Table 2).

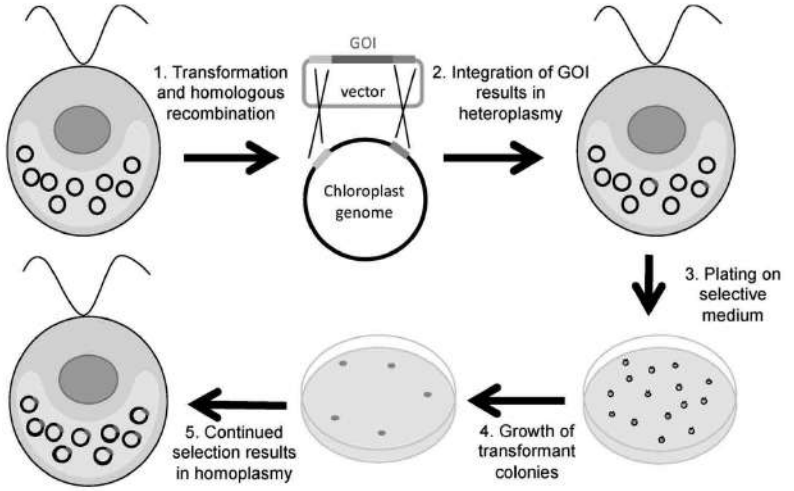


Figure 36. Plastome transformation in *C. reinhardtii*. Source: "Selectable Markers and Reporter Genes for Engineering the Chloroplast of *Chlamydomonas reinhardtii*" by L. Esland, M. Larrea-Alvarez, S. Purton, 2018, *Biology*, 7, p. 46. GOI, gene of interest.

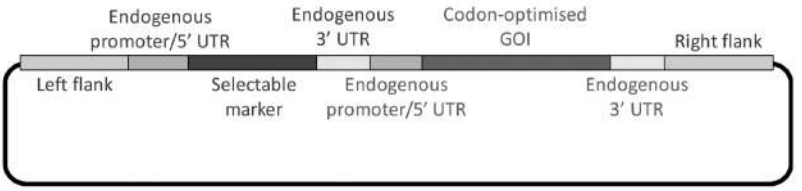


Figure 37. Plasmid vector for transformation. Source: "Selectable Markers and Reporter Genes for Engineering the Chloroplast of *Chlamydomonas reinhardtii*" by L. Esland, M. Larrea-Alvarez, S. Purton, 2018, *Biology,* 7, p. 46.

Table 2. *Sequences used for transgene expression*

GENE EXPRESSION	
Promoters/5'UTRs	*atpA, psaA exon 1, psbD, petB, chlL*
3'UTRs	*rbcL, atpB, psbA*
SELECTION MARKERS	
rrnS, rrnL point mutations	Resistance to spectomycin and erythromycin
aadA	Resistance to spectinomycin
aph6	Resistance to kanamycin
psbH	Photosynthetic restoration
GFP	Fluorescent protein
gusA	β-glucuronidase activity
codA	5-fluorocytosine sensitivity
TARGETING SEQUENCES (FOR HOMOLOGOUS RECOMBINATION)	
patpint-cg11 (atpB-int)	*atpB* 3'UTR inverted repeat
pLM7	*psbA* and 5S/23S
p322	*psbA* 16S rRNA
p71	*tscA* and inverted repeats
p72B	*psbH – psbN*

Note. Adapted from "Establishing *Chlamydomonas reinhardtii* as an Industrial Biotechnology Host" by M. A. Scaife, G. T. Nguyen, J. Rico, D. Lambert, K. E. Helliwell, A. G. Smith, 2015, *The Plant Journal*, 82, pp. 532-546.

Streptomycin and spectinomycin target the 70S ribosome found in both bacteria and chloroplasts. Therefore, it's common to use a portable cassette that confers resistance to these drugs. The *aadA* gene, which encodes for an aminoglycoside adenyltransferase catalyzing the adenylation and subsequent inactivation of these drugs, is often employed for this purpose. When fused to chloroplast endogenous sequences, this gene is functionally expressed and serves as a dominant marker for selection. Similarly, the *aphA6* gene, when linked to appropriate *cis* elements, is utilized for selection as it encodes an aminoglycoside phosphotransferase conferring resistance to kanamycin, another antibiotic targeting the 70S ribosome. While other drugs like erythromycin and tetracycline have been explored, their results have been limited. Although expression of the associated resistance mechanism was achieved, direct selection using these drugs was not successful. Additionally, attempts with other genes such as *arr-2* and *cat*, which confer resistance to rifampicin and chloramphenicol respectively, have also yielded unsuccessful results.

A potential alternative to expand the repertoire of selection markers is the synthesis of bifunctional enzymes. For example, the production of an Aad-Aph enzyme would allow the inactivation of aminoglycosides by different mechanisms, namely, adenylation and phosphorylation. However, there are various disadvantages of using selection methods based on antibiotic resistance. First, the presence of antibiotic resistance genes is undesirable in genetically modified organisms (GMO), as they present potential hazards regarding the environmental dissemination of such traits. Second, producing an enzyme constantly represents a burden for the system, especially if selection has been achieved and the gene is no longer needed. Furthermore, the use of *cis* elements to drive the expression of markers imposes a limitation due to competition for *trans* factors needed for transgene expression. Third, resistance to these drugs can arise naturally and is associated with point mutations in genes encoding the ribosomal RNA of the large and small subunits. Mutations in *rrn* S of the 16S subunit confer resistance to streptomycin and spectinomycin, whereas mutations in *rrn* L of the 23S subunit confer resistance to erythromycin. These point mutations have been exploited as endogenous markers, although they are not portable and the integration of the marker along with the gene of interest has proved troublesome. Herbicide selection is another option encompassing endogenous resistance that has been examined, notwithstanding the concerns raised by this type of selection. In this case, mutations in the *psbA* gene that encodes for the D1 protein of PSII confer resistance to various herbicides such as metribuzin or phenmedipham.

As detailed in Chapter 5, *C. reinhardtii* exhibits robust growth when supplied with acetate as a carbon source, leading to the identification of various photosynthetic mutants in the scientific literature. These mutants offer an alternative approach for selection, as their impaired photosynthesis can be restored by reintroducing wild-type versions of the mutated genes. Selection is subsequently conducted on minimal medium devoid of acetate. This method has been reported to facilitate the attainment of homoplasmy more efficiently compared to those relying on antibiotic selection. This not only expedites the production of stable transformants but also reduces the likelihood of

spontaneous reversion to phototrophy, thereby minimizing the occurrence of "false positives." Moreover, this approach enables the generation of marker-free transformants, a significant advantage (Figure 38). Mutants lacking functional versions of genes such as *tscA*, *psbA*, and *atpB* are particularly useful for these purposes. For instance, a transformation event could be performed to disrupt certain photosynthetic genes using the *aadA* cassette. Knocking out these sequences generates a strain incapable of photosynthesis, which can serve as a recipient strain for subsequent transformation events. In this scenario, a functional version of the altered gene is introduced at a silent site along with the gene of interest, followed by selection on minimal medium. Illustrative examples of this methodology include the *petB*::*aadA* and *psbH*::*aadA* constructs.

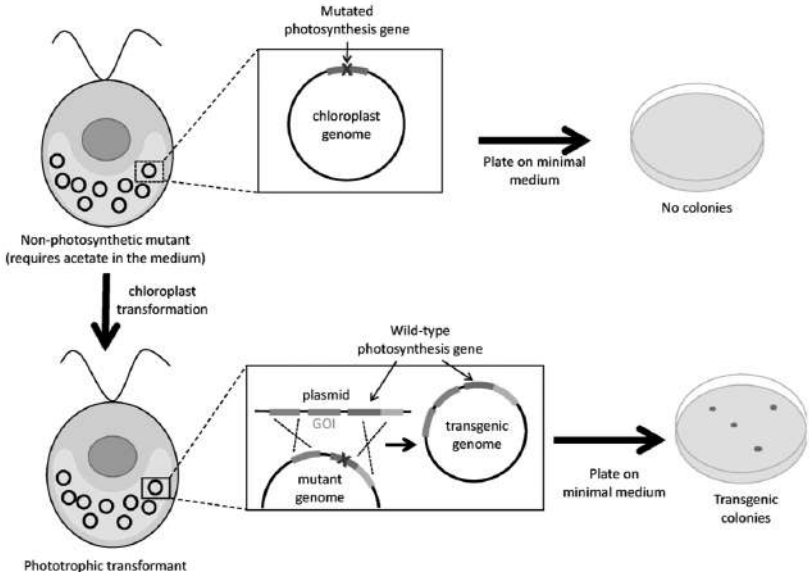


Figure 38. Photosynthetic selection. Source: "Selectable Markers and Reporter Genes for Engineering the Chloroplast of *Chlamydomonas reinhardtii*" by L. Esland, M. Larrea-Alvarez, S. Purton, 2018, *Biology*, 7, p. 46.

In addition to the methods discussed, alternative techniques have been explored for selecting transformants. One such approach involves selecting based on arginine synthesis. Additionally, phosphorus availability has been

utilized as a selection criterion. Bacteria and eukaryotes, which utilize phosphate (PO_4) for phosphorus, they are unable to use phosphite (PO_3). By introducing the phosphite oxidoreductase gene (*ptxD*) into the plastome, transformants can be selected in growth media containing only PO_3. Furthermore, efforts have been made to develop strategies for recycling selectable markers, particularly in the context of antibiotic resistance genes like *aadA*. After successful transformation, the selective pressure used for marker selection is removed, leading to the marker's loss. However, implementing these approaches has proven challenging due to the necessity of co-transformation and repeated streaking.

Besides selectable markers, reporter genes play a crucial role in chloroplast engineering by encoding proteins that exhibit detectable phenotypes post-transformation. Selectable marker genes can also serve as reporter genes, as they enable the identification of transformed cells. Recombinant fluorescent proteins have been successfully expressed in the chloroplast, allowing for fluorescence detection under specific illumination, typically using short-wave light in fluorescence microscopy. One of the most widely used fluorescent proteins is green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria*, which emits green fluorescence upon excitation. While various GFP derivatives have been developed to expand the range of visible light, some have been tested in *C. reinhardtii* chloroplasts with limited success in producing detectable fluorescence levels. However, luciferase enzymes have been expressed successfully, enabling the detection of bioluminescence, which has been utilized in studies monitoring changes in gene expression. Another approach involves the scoring of yellow/green phenotypes characteristic of chlorophyll-deficient mutants. Unlike angiosperms, green algae can synthesize chlorophyll even in the absence of light. Therefore, when grown in dark conditions, they maintain a green appearance. Plastid-located genes encoding an oxidoreductase enzyme involved in chlorophyll precursor synthesis, such as *chlL*, *chlB*, and *chlN*, can serve as reporters. Inactivation of these genes disrupts light-independent chlorophyll synthesis, causing dark-grown colonies to exhibit a pale phenotype. Thus, successful transformation

results in colonies growing pale in the absence of light, providing a visible indicator of transformation success. The tools mentioned above have enabled the expression of approximately 100 proteins from the plastome, with more than 40 of them possessing therapeutic properties (Table 3).

Table 3. *Valuable proteins produced in the chloroplast*

SUBUNIT VACCINES	
Protein	**Detail**
CTB-VP1	CTB adjuvant with foot-and-mouth disease virus structural protein VP1 (farm-animal pathogen)
E2	E2 structural protein of classical swine fever virus (porcine pathogen)
p57	*Renibacterium salmoninarum* protein p57, causing bacterial kidney disease in salmonid fish
VP28	White spot syndrome virus envelope protein VP28 (pathogen of crustaceans)
CTB-D2	*Staphylococcus aureus* D2 fibronectin-binding domain with CTB adjuvant
AcrV and VapA	Antigens from a bacterial pathogen of salmonids (*Aeromonas salmonicida*)
Pfs25 and Pfs28	Surface protein antigens from the malarial parasite, *P. falciparum*
CTB-Pfs25	CTB adjuvant fused to the Pfs25 surface antigen of *P. falciparum*
Pfs48/45	*P. falciparum* surface protein antigen
E7GGG	A mutated, attenuated form of the E7 oncoprotein from human papilloma virus type 16
E7GGG-AadA	E7GGG fused to the bacterial spectinomycin resistance enzyme, AadA
MPT64	Secreted antigen of *Mycobacterium tuberculosis*
HA	Haemagglutinin of avian influenza virus H5
CTB-p210	CTB adjuvant fused to the p210 epitope of ApoB100, the main apolipoprotein in low density lipoproteins associated with atherosclerosis
AUTOANTIGENS	
hGAD65	Human glutamic acid decarboxylase
hIL4	Human interleukin 4
ALLERGENS	
Ara h 1 core domain and Ara h 2	Major peanut allergens
Bet v 1	Major birch pollen allergen
MONOCLONAL ANTIBODIES	

NANOBODIES	
Protein	**Detail**
V_HH	Variable domain of camelid heavy chain-only antibodies targeting botulinum neurotoxin
IMMUNOTOXINS	
aCD22CH23PE40	Chimeric antibody to B-cell surface antigen CD22 fused to the enzymatic domain of exotoxin A from *Pseudomonas aeruginosa*
aCD22CH23Gel	Chimeric antibody to B-cell surface antigen CD22 fused to 80S ribosome-inactivating protein gelonin from *Gelonium multiflorum*
ANTIBODY MIMICS	
10FN3	Tenth binding domain of human fibronectin type III
14FN3	Fourteenth binding domain of human fibronectin type III
GROWTH FACTORS	
VEGF	Vascular endothelial growth factor
hGH	Human growth hormone
GUT–ACTIVE PROTEINS	
M-SAA	Bovine mammary-associated serum amyloid
AppA	Phytase from *E. coli*
NCQ	Chimeric protein comprising 20 known bioactive peptide sequences from milk proteins
PhyA-E228K	Phytase from Aspergillus niger
WOUND–HEALING FACTORS	
HMGB1	High-mobility group protein B1
ANTI–BACTERIALS	
Cpl-1 and Pal	Endolysins from bacteriophage of *Streptococcus pneumoniae*
CANCER CELL THERAPEUTICS	
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

(mAb) HSV8-lsc	Large single-chain (lsc) antibody against glycoprotein D of herpes simplex virus	**ANTI–HYPERSENSITIVE PEPTIDES**	
83K7C	Human IgG1 antibody against anthrax protective antigen 83	VLPLP	Chimeric protein containing anti-hypertensive peptides

Note. Adapted from "The Algal Chloroplast as a Synthetic Biology Platform for Production of Therapeutic Proteins" by Y. M. Dyo, S. Purton, 2018, *Microbiology*, 164, pp. 113-121.

The majority of these proteins consist of single subunits, requiring the introduction of only one transgene. However, there are instances where the expression of multiple genes is necessary. Most of these recombinant proteins are soluble and accumulate in the stroma, although some are expressed in the thylakoid lumen, while others are associated with membranes. Table 3 illustrates several examples of therapeutic proteins produced in the chloroplast, and we are focusing on four specific cases. First, *C. reinhardtii* is considered edible and safe for consumption, making it a potential candidate for oral delivery of protein vaccines, particularly for farmed animals like poultry or fish. Various antigens for viral, bacterial, and parasitic pathogens have been synthesized in the organelle, many of which can provoke an immune response in animal models. These recombinant vaccines are typically fused (at N-terminal sites) to the cholera toxin B subunit, which serves as an adjuvant. These pentameric structures bind to the GM1 ganglioside receptor on gut epithelial cells. The recombinant vaccines are retained within the cell in the chloroplast and have demonstrated stability at room temperature for extended durations. This stability is particularly advantageous, as the whole dried cell can be integrated into animal feed, simplifying vaccine delivery. Moreover, this method could reduce vaccine distribution costs, as expensive cold chain transportation may become unnecessary. Additionally, the vaccines are protected not only by the chloroplast and cellular membranes but also by the cell wall, offering natural means to encapsulate the epitopes and safeguard them until they reach the gut epithelium.

Secondly, commercial monoclonal antibodies are both costly and in limited supply. Typically, they are manufactured using mammalian cell cultures due to the presence of disulfide bonds in these proteins, which are challenging to

produce in prokaryotic systems. While the chloroplast lacks the machinery for protein glycosylation, successful expression of monoclonal antibodies has been achieved in this organelle. Despite being aglycosylated, these chloroplast-produced antibodies retain their ability to bind to their targets. The genes encoding the heavy and light chains of the antibodies were introduced and expressed from the plastome. Chloroplast-derived antibodies effectively bound to antigens from *Bacillus anthracis*, the causative agent of anthrax. Immunotoxins serve as another example of complex proteins produced in the chloroplast. These chimeric molecules consist of two domains: one linked to a cytotoxic protein and the other to an antibody. Due to their cytotoxic nature, their synthesis in eukaryotic systems poses challenges and can even be lethal. In prokaryotes, issues arise with protein folding and assembly. Nonetheless, two immunotoxins were successfully synthesized in the chloroplast. One contained a single-chain antibody linked to a bacterial exotoxin, while the other was linked to a ribosome-inactivating protein derived from plants. Both immunotoxins targeted the CD22 receptor found on B cells. In vitro experiments demonstrated that the plastome-encoded proteins effectively bound to B cells. Furthermore, when applied to mice carrying human B-cell tumors, these chloroplast-produced immunotoxins which extended the animals' overall lifespan. These results not only underscore the chloroplast's capability to properly fold and assemble complex proteins but also demonstrate that the 70S ribosome remains unaffected by ribosome-inactivating proteins, a concern in other production platforms. Undoubtedly, the chloroplast emerges as an attractive compartment for the production of complex proteins.

Thirdly, chloroplasts have emerged as valuable platforms for the production of novel antibiotics. Endolysins are antibacterial proteins produced by bacteriophages, capable of breaking down the cell wall of their host bacteria during the lytic cycle, facilitating the release of phage progeny. Due to the high specificity of phages and their proteins for their target bacteria, the development of resistance is rare, making endolysins a promising alternative as novel antibiotics. Recombinant forms of endolysins produced in chloroplasts have demonstrated effectiveness comparable to those derived from natural phages

when targeting bacterial cultures and biofilms. The chloroplast environment is conducive to the synthesis of these proteins, resembling the bacterial milieu where endolysins are naturally produced. Unlike prokaryotic platforms, chloroplasts lack a peptidoglycan wall that could limit their overexpression. Two endolysins, Pal and Cpl1, designed to target *Streptococcus pneumoniae*, have been successfully expressed from the chloroplast genome. Crude cell lysates containing these recombinant proteins have exhibited potent lytic activity against *S. pneumoniae*. Consequently, they hold promise as ingredients in antibacterial creams or sprays intended for combating topical infections.

Human growth hormone (hGH) represents another therapeutic protein successfully produced in the chloroplast. The demand for this polypeptide is steadily increasing due to its application in treating growth deficiencies resulting from insufficient hormone levels in the body. hGH, naturally synthesized by the pituitary gland, undergoes post-translational modifications such as N-terminal methionine removal and intrapeptide disulfide bond formation. While *E. coli* is commonly utilized for hGH production, achieving correct folding requires targeting proteins to the periplasm. In the chloroplast, a codon-optimized version of the hGH gene was linked to endogenous chloroplast components promoting expression before being introduced into the plastome. Although expression levels were not notably high, crude cell lysates demonstrated activity by stimulating cells of a rat lymphoma line. These findings, alongside others, underscore the viability of the chloroplast as a platform for producing pharmaceuticals like hGH and other bioactive peptides such as insulin. Furthermore, allergens have been synthesized within the chloroplast, offering promise in treating food allergies like peanut allergies through oral, sublingual, and epicutaneous delivery routes. However, the use of allergens entails risks of adverse reactions as they derive from proteins found in peanut extracts. Thus, their use is only recommended in specific cases. Recombinant allergens, in contrast, are purified to eliminate contact with peanut proteins, and modifications are made to mitigate allergic reaction severity. Notably, chloroplast-derived allergens from *C. reinhardtii* have demonstrated efficacy in protecting against peanut-induced anaphylaxis in mice.

Phytase serves as a crucial additive in animal feed, particularly in plant-derived feeds where around 80% of phosphorus exists as phytate. Monogastric animals like swine, poultry, or fish lack phytases, enzymes necessary for phytate digestion, requiring the supplementation of exogenous enzymes to enhance animal growth. Recombinant proteins produced in *C. reinhardtii* and fed to broiler chickens proved effective for reducing animal phytate excretion. Remarkably, the cost of producing phytases in microalgae aligns with that of commercial suppliers. These examples underscore the potential of microalgae, particularly *C. reinhardtii*, as a straightforward, cost-effective, and environmentally benign platform. Transplastomic engineering offers advantages such as efficient folding and accumulation of complex proteins, absence of contaminants and infectious agents, making it ideal for pharmaceutical production and storage. Nonetheless, the full potential of this approach could be further enhanced through synthetic biology tools enabling the creation of designer strains.

The advancement of genetically validated part libraries, along with streamlined assembly techniques, holds the promise of reengineering the plastome by eliminating non-essential genes and reorganizing the remaining endogenous ones alongside transgenes in various loci. Synthetic biology principles offer alternatives for enhancing the production yield of recombinant proteins. Currently, promoters, as well as 5' and 3' UTRs from endogenous genes, are utilized. For example, the promoter of the 16S rRNA and the UTR regions of photosynthetic genes have demonstrated effectiveness in driving foreign gene expression. However, 5' UTRs present a bottleneck due to the chloroplast's feedback regulation mechanism that restricts the overaccumulation of photosynthetic units and the competition for *trans* factors necessary for RNA stability and translation. Designing synthetic promoters and 5' UTRs that evade native regulation pathways can elevate the levels of recombinant proteins. Progress in these areas is imperative for attaining the requisite protein yields for commercial applications.

Undoubtedly, genetic engineering of the chloroplast has become feasible and commonplace in various research institutions and industrial settings.

However, while the introduction of single genes is relatively straightforward, there is currently no standardized approach for achieving higher levels of complexity involving multiple gene insertions. Future studies are expected to demonstrate the feasibility of metabolic engineering within the organelle, potentially enabling the introduction of novel reactions such as nitrogen fixation or alternative carbon fixation pathways. Despite the advantages offered by chloroplast engineering, there are also limitations to consider. Firstly, *C. reinhardtii*, being a unicellular organism adapted to a mixotrophic lifestyle in resource-scarce soil and water environments, imposes constraints on the introduction of foreign metabolic pathways into the organelle. Secondly, unlike certain plant tissues, chloroplasts do not undergo differentiation even under optimal conditions, limiting the exploration of specific plant pathways. Nonetheless, the progress in chloroplast engineering has been significant for biotechnology and has laid the groundwork for similar strategies targeting other microalgae species. Future efforts may focus on developing these methodologies in commercially relevant species such as *C. vulgaris* or *D. salina*, with the aim of enabling large-scale and cost-effective production of therapeutic compounds in microalgae.

Chapter 8

Conclusion

Our discussion began by emphasizing the profound impact of cyanobacteria on the development of life on Earth. Their photosynthetic activity not only facilitated the rise of aerobic organisms through the production of oxygen but also continues to hold significance today, both ecologically and in the realm of biotechnology. Photosynthesis, the process through which cyanobacteria harness solar energy to produce chemical energy and reducing power for the Calvin cycle, involves a complex series of reactions. Initially, photons excite specific chlorophyll molecules, leading to the transfer of electrons along an electron transport chain. This electron movement generates an electrical current that drives the translocation of hydrogen ions across membranes, establishing a proton gradient that powers ATP synthesis. Additionally, electrons are utilized to reduce molecules, ultimately yielding NADPH from $NADP^+$ and H^+. Both ATP and NADPH are pivotal in phosphorylating and reducing 3-phosphoglycerate into glyceraldehyde 3-phosphate, a precursor in the Calvin cycle. This cycle involves the carboxylation of ribulose 1,5-bisphosphate by the enzyme RUBISCO. Some glyceraldehyde 3-phosphate is further utilized, along with ATP, to regenerate the acceptor and sustain the cycle, while others are condensed into hexoses, essential building blocks for complex polysaccharides. Cyanobacteria possess specialized compartments, namely thylakoids and carboxysomes, dedicated to ATP/NADPH production and carbon fixation, respectively. All the genetic information required for the assembly and functioning of these single-celled organisms is encoded within their genome, with some additional instructions carried by plasmids. A core

set of genes encompassing DNA replication, gene expression, photosynthesis, carbon fixation, and respiration is essential for cyanobacterial function. Moreover, cyanobacteria are considered the ancestors of modern chloroplasts, the defining feature of photosynthetic eukaryotes. The chloroplast, being a prokaryotic-like environment within a eukaryotic cell, holds immense potential for biotechnology applications. This unique setup offers opportunities to introduce bacterial pathways, such as nitrogen fixation, into algae or plants, expanding the repertoire of possible applications in various fields.

As outlined in Chapter 3, the chloroplast serves as a hub for crucial metabolic activities such as photosynthesis, amino acid and nucleic acid synthesis, and the synthesis of essential fatty acids. In plants, plastids exhibit specialization for specific functions, such as starch accumulation in amyloplasts. Inherited from their cyanobacterial ancestors, chloroplasts feature thylakoids housing protein complexes responsible for generating NADPH and ATP. Notably, photosystems within these thylakoids capture light energy, with pigments like carotenoids facilitating energy transfer and dissipation. Following light absorption, ATP and NADPH are released into the stroma, where they fuel CO_2 fixation. The chloroplast harbors its own genetic material organized akin to bacterial genomes, including genes arranged in operons that yield polycistronic mRNAs. Transcription is facilitated by plastid-encoded polymerases (PEP), resembling bacterial enzymes, which necessitate sigma cofactors, some of which are nuclear-encoded. Additionally, plants feature another RNA polymerase localized in the chloroplast, responsible for transcribing housekeeping genes and also originating from the nucleus. Post-transcriptional regulation in chloroplasts encompasses RNA stabilization and processing, with translation facilitated by bacterial-like ribosomes. These processes are governed by nuclear factors, underscoring the intricate interplay between the chloroplast's bacterial ancestry and the host plant's nuclear genome.

Algae exhibit several key characteristics, including a well-organized nucleus, distinctive pigment compositions, storage polysaccharides, and the presence of flagella. They encompass three primary lineages: glaucophytes,

rhodophytes, and chlorophytes, each containing plastids derived from a bacterial ancestor with unique traits. Muroplasts and rhodoplasts produce phycobilins, while chloroplasts have lost this ability. Additionally, the site of starch accumulation varies; chlorophytes store it within the organelle, while others store it in the cytoplasm. Heterotrophs ingested chlorophytes and rhodophytes, giving rise to cells with secondary chloroplasts. For instance, euglenoids and chlorarachniophytes harbor green algae-derived plastids, while cryptophytes, haptophytes, heterokontophytes, and dinoflagellates contain organelles from red algae. Apicomplexa provides a fascinating example of photosynthetic organisms evolving into parasites, losing their carbon fixation capabilities, and retaining vestigial chloroplasts known as apicoplasts, which aid in invasion. Notable parasites within this group include malaria and toxoplasmosis agents. Other instances of endosymbiosis include a cyanobiont engulfed by an ameboid, leading to the photosynthetic species *Paulinella*, and a ciliate hosting a photosynthetic alga and a sulfur bacterium. Algae have long been utilized by humans, primarily in food culture, but have more recently found applications in pharmaceutical compound production, bioremediation, animal feed, pollution control, and biofuel production. Genetic engineering of both nuclear and chloroplast genomes has been achieved in many algae species, although this field is more advanced in green algae compared to other microalgae.

C. reinhardtii is a versatile species found in various habitats, spanning temperate, tropical, and subtropical regions, inhabiting both soil and aquatic environments such as lakes and oceans. Typically dwelling near the surface to access light, these microalgae possess flagella connected to an eyespot, aiding in movement and environmental sensing. Beyond being photoautotrophic, they can also sustain heterotrophic growth using 2-carbon molecules like acetate. This adaptability has made *C. reinhardtii* an invaluable model organism, facilitating the study of fundamental biological processes. Its haploid vegetative cells enable straightforward expression of mutant phenotypes, while its rapid growth in mixotrophic conditions has been instrumental in studying photosynthetic genes and electron transport chain dynamics. Furthermore, the

discovery of light-gated ion channels in the eyespot has significantly advanced the field of optogenetics, in which these receptors are used to modulate membrane potentials and regulate processes such as neuronal activity. Today, *C. reinhardtii* continues to unravel key biological phenomena, including basal body and centriole development, pyrenoid structure, light acclimation, the cell cycle, organism interactions, and ecosystem dynamics. With the advent of biotechnology, the species has seen further advancements, from genome sequencing to the expression of foreign proteins in its cytoplasm, mitochondria, and chloroplasts, opening up new avenues for research and applications.

As discussed, the plastome, a circular DNA molecule, houses approximately a hundred genes categorized into three groups. Firstly, genes associated with the photosynthetic machinery, including those encoding components of photosystems. Secondly, genes related to replication and expression machinery, such as DNA and RNA polymerases, ribosome-associated proteins, rRNAs, and tRNAs. Lastly, sequences encoding proteins crucial for maintaining stability in the aforementioned factors. Transcription primarily occurs from bacterial-like promoters by a single RNA polymerase encoded in the plastid genome, generating both monocistronic and polycistronic RNAs, with subsequent post-transcriptional modifications, including intron splicing. mRNA degradation plays a pivotal role in processes like nucleotide recycling, damaged molecule removal, and translation rate regulation. The ribosome exhibits prokaryotic traits alongside organelle-specific constituents, with counterparts for proteins found in *E. coli* and spinach subunits identified. mRNA contains specific sequences facilitating interaction with factors imported from the cytoplasm, ensuring proper translation. Notably, crosstalk between nuclear and plastid genomes is essential for maintaining metabolic balance within the organelle. This adaptable system holds promise for engineering endeavors due to several advantages. Homologous recombination facilitates the introduction of foreign genetic material, yielding relatively high levels of recombinant protein expression with correct disulfide bond formation. Additionally, diverse metabolic pathways occurring within the organelle can be redirected for the synthesis of valuable metabolites. Engineered strains harness light as an energy source to drive the production of commercially relevant molecules.

The chloroplast genetic system blends prokaryotic traits inherited from cyanobacteria with unique features evolved within the organelle. This relatively straightforward system has been harnessed for genetic engineering to produce valuable proteins. Foreign genetic material is integrated into the plastome via homologous recombination, facilitated by sequences within the plasmid vector containing the transgenes. Additionally, plasmids must harbor chloroplast endogenous sequences essential for genetic expression, including promoters and untranslated regions (UTRs). Codon optimization of transgenes is also essential to achieve optimal expression levels. Various methods, such as electroporation, biolistics, or agitation with glass beads, can be employed to introduce the vector. The aim is to create pores in both the cellular and chloroplast membranes, enabling plasmid passage into the organelle. Selection of transformants is crucial and typically involves applying selective pressures. Recombinant proteins can be directed to the stroma, membranes, or even the thylakoid lumen, depending on their intended function and application. This versatility allows for the targeted production of proteins with diverse functionalities within the chloroplast.

Over a hundred proteins have been successfully synthesized in the chloroplast, with approximately 40 of them demonstrating therapeutic properties. In particular, vaccines targeting bacterial, viral, or parasitic pathogens have been produced, eliciting immune responses in animal models. Furthermore, endolysins, which are bacteriophage-derived proteins capable of digesting bacterial cell walls, have been expressed in the chloroplast, demonstrating effectiveness against *S. pneumoniae* growth in vitro. Monoclonal antibodies directed against *B. anthracis* have also been synthesized, effectively detecting their target. Additionally, immunotoxins have been engineered in the chloroplast, accurately targeting desired receptors and exerting the intended effects. In addition to these achievements, the chloroplast has been utilized for the production of other vital molecules such as phytases and bioactive peptides like human growth hormone. Synthetic biology holds promise in further enhancing the chloroplast's potential as a recombinant expression platform by developing DNA libraries and novel vector assembly techniques. Designing

synthetic endogenous sequences for expression could significantly boost recombinant protein yields by eliminating competition with native counterparts for *trans* factors and avoiding negative feedback regulation that limits protein accumulation. The methodologies established for *C. reinhardtii* serve as a solid foundation for engineering chloroplasts in commercially relevant microalgae like *Dunaliella* or *Chlorella*. By exploiting these advancements, researchers can continue to expand the capabilities of chloroplast-based biotechnology for various applications.

Chloroplast engineering in *C. reinhardtii* has undergone significant advancements, transitioning from a tool primarily used for studying fundamental biological processes to a platform for producing a diverse array of valuable products within the organelle. Initially, genetic engineering focused on introducing single transgenes encoding for single subunit proteins, yielding antimicrobials, bioactive peptides, and vaccines, among others. In recent years, however, the focus has shifted towards engineering complex pathways involving multiple transgenes. This shift towards metabolic engineering opens up exciting possibilities for adapting foreign pathways to the chloroplast's metabolism, potentially enabling processes like nitrogen fixation. These developments have revolutionized our perception of *C. reinhardtii*'s biotechnological applications, shedding light on the intricate regulatory crosstalk that governs the organelle. As research progresses, our understanding of the chloroplast deepens, bringing us closer to fully harnessing its potential as a compact, efficient photosynthetic factory. Further research promises to unveil even more opportunities for leveraging this microalga in diverse biotechnological endeavors.

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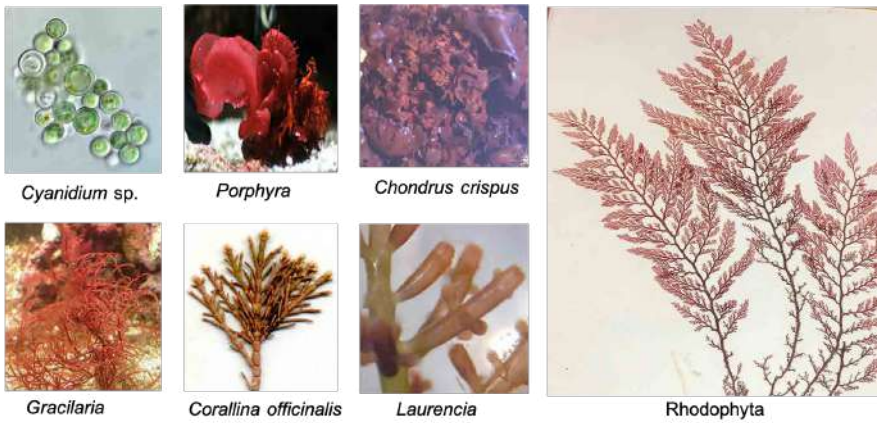
Annexes

Glaucocystis sp.

Cyanophora paradoxa

Annex 1. Glaucophytes. Adapted from: File:Glaucocystis sp.jpg. (2022, May 18). *Wikimedia Commons*. Retrieved October 1, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Glaucocystis_sp.jpg&oldid=656957831; File:Woelfib cyanphoraparadoxa teilungsfigur 1 0632002 img 54414492 ude 20131024233254 small.jpg. (2022, June 5). *Wikimedia Commons*. Retrieved October 1, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Woelfib_cyanphoraparadoxa_teilungsfigur_1_0632002_img_54414492_ude_20131024233254_small.jpg&oldid=661841227 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).



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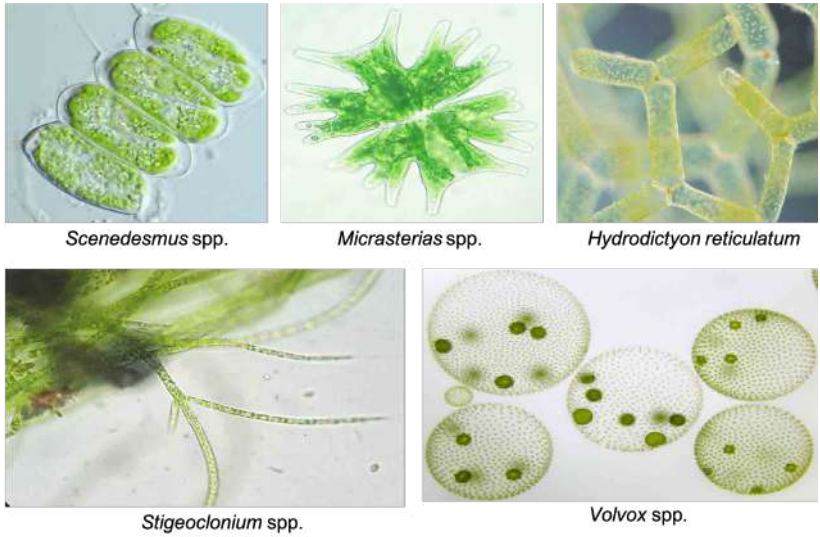

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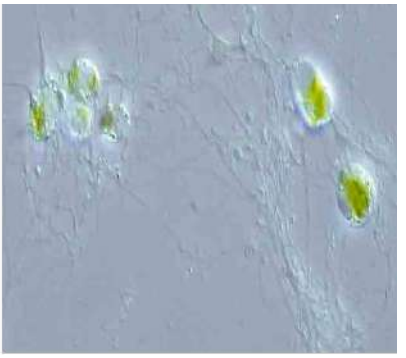
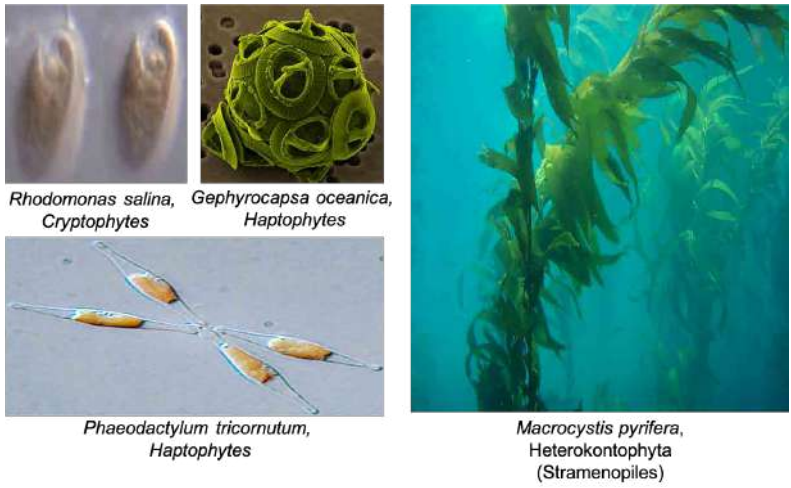
Euglena



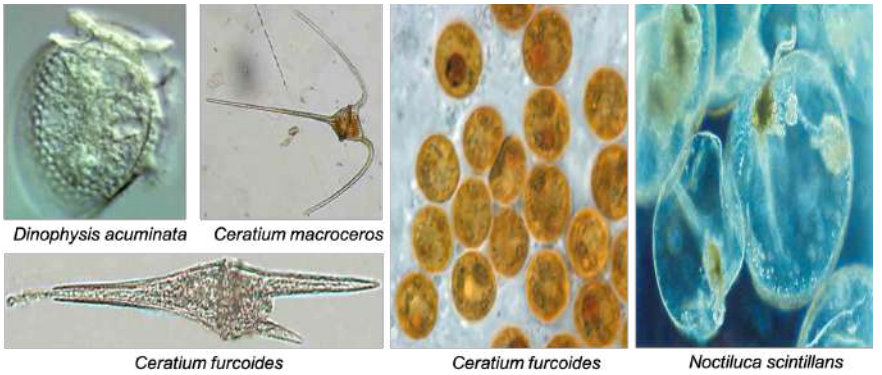
Chlorarachnion reptans

Annex 4. Algae with green secondary plastids. Source: File:Two Euglena. jpg. (2020, December 23). *Wikimedia Commons*. Retrieved October 5, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Two_

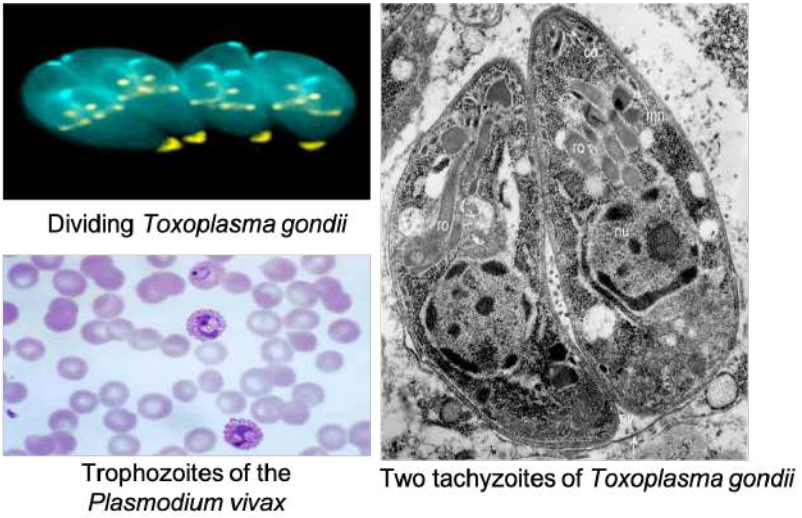
Euglena.jpg&oldid=520536624; File:Chlorarachnion reptans.jpg. (2020, September 13). *Wikimedia Commons*. Retrieved October 5, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Chlorarachnion_reptans.jpg&oldid=457260575 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).



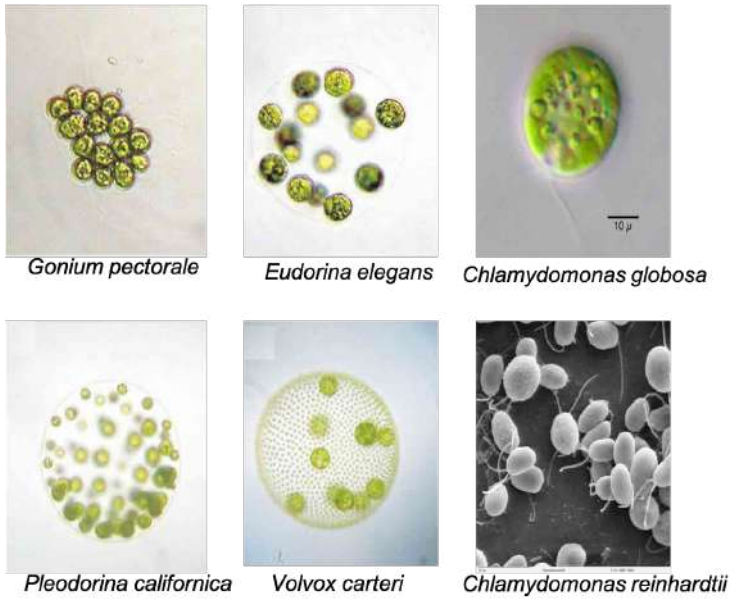
Annex 5. Algae with red secondary plastids. Source: File:Rhodomonas salina CCMP 322.jpg. (2020, October 25). *Wikimedia Commons*. Retrieved October 6, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Rhodomonas_salina_CCMP_322.jpg&oldid=500816533; File:Gephyrocapsa oceanica color.jpg. (2023, August 26). *Wikimedia Commons*. Retrieved October 6, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Gephyrocapsa_oceanica_color.jpg&oldid=796235137; File:Phaeodactylum tricornutum.png. (2020, September 16). *Wikimedia Commons*. Retrieved October 6, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Phaeodactylum_tricornutum.png&oldid=461192239; File:Sanc0063 - Flickr - NOAA Photo Library.jpg. (2023, May 15). *Wikimedia Commons*. Retrieved October 6, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Sanc0063_-_Flickr_-_NOAA_Photo_Library.jpg&oldid=763534045 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).



Annex 6. Dinoflagellates. Dinoflagellate. (2023, September 16). In *Wikipedia, The Free Encyclopedia*. Retrieved October 7, 2023, from https://en.wikipedia.org/w/index.php?title=Dinoflagellate&oldid=1175711794 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).



Annex 7. Apicomplexa. Source: Apicomplexa. (2023, August 24). In *Wikipedia, The Free Encyclopedia*. Retrieved October 7, 2023, from https://en.wikipedia.org/w/index.php?title=Apicomplexa&oldid=1172024726 (This file is licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).



Annex 8. Volvocales. Source: File:Volvocales.png. (2020, September 8). *Wikimedia Commons*. Retrieved October 8, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Volvocales.png&oldid=452192479; File:Chlamydomonas globosa - 400x (13263097835).jpg. (2022, November 27). *Wikimedia Commons*. Retrieved October 8, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Chlamydomonas_globosa_-_400x_(13263097835).jpg&oldid=710014642; File:Chlamydomonas6-1.jpg. (2020, September 12). *Wikimedia Commons*. Retrieved October 8, 2023 from https://commons.wikimedia.org/w/index.php?title=File:Chlamydomonas6-1.jpg&oldid=456911167 (These files are licensed under the Creative Commons Attribution-Share Alike 4.0 Unported license. https://creativecommons.org/licenses/by-sa/4.0/deed.en).

Annex 9.

DIFFERENCES	
Prokaryotes	**Eukaryotes**
Polymerase I (involved in synthesis, proofreading, repair and RNA primers removal)	Polymerase α (a polymerizing enzyme)
Polymerase II (a repair enzyme)	Polymerase β (a repair enzyme)
Polymerase III (main polymerizing enzyme)	Polymerase γ (mitochondrial DNA synthesis)
Polymerase IV and V (repair enzymes under unusual conditions)	Polymerase δ (main polymerizing enzyme)
	Polymerase ε (unknown function)
Polymerases = exonucleases	Not all polymerases are exonucleases
One origin of replication	Several origins of replication
Okazaki fragments 1000–2000 residues long	Okazaki fragments 150–200 residues long
No proteins complexed to DNA	Histones complexed to DNA
RNA Polymerase consists of 4 subunits and sigma factor; occurs in 1 type	RNA Polymerase occur in 5 types (RNA Polymerase I, II, III, IV, and V)
Core enzyme contains 5 subunits	Core enzyme contains 10–20 subunits
RNA Polymerase size is around 400 kDa	RNA Polymerase size is around 500 kDa
Synthesizes polycistronic RNA	Synthesizes monocistronic RNA
Transcription is regulated using different types of sigma factors	Transcription is regulated by the presence of different types of RNA polymerases
Transcription and translation are continuous process and occurs simultaneously in the cytoplasm	Transcription and translation are separate process, transcription occurs in the nucleus and translation in the cytoplasm
mRNA 5'end is immediately available for translation	The primary transcript is processed after transcription and then transported to the cytoplasm where only the cytoplasmic ribosomes can initiate translation
Ribosome 70S type (composition of 50S larger subunit and 30S smaller subunit)	Ribosome 80S type (composition of 60S larger subunit and 40S smaller subunit)
Larger subunit of ribosome with two rRNA molecules 5S and 23S rRNA	Larger subunit of ribosome with three rRNA molecules 5S, 5.8S and 28S rRNA
Smaller subunit of ribosome (30S) with 16S rRNA	Smaller subunit of ribosome (40S) with 18S rRNA
Smaller subunit of ribosome with 21 proteins	Smaller subunit of ribosome with ~33 proteins
Larger subunit of ribosome with 36 proteins	Larger subunit of ribosome with ~49 proteins
Ribosome mass 2700 kD	Ribosome mass 4200 kD
Absence of endoplasmic reticulum (ER), protein synthesizing ribosome freely distributed in the cytoplasm	Presence of ER and protein synthesizing ribosome usually attached to the ER
mRNA can act as the template for the synthesis of many polypeptides	mRNA can act as the template for a single polypeptide
1 type of translation initiation mechanism (cap independent)	2 types of translation initiation mechanism (cap depended and cap independent)
May have many start sites and Shine-Dalgarno sequences (SD) along with the mRNA	Only 1 start side located towards the 5' region of mRNA
SD sequence present 8 nucleotides upstream of start codon (SD sequence act as the ribosome binding site)	SD sequence is absent in mRNA
Kozak sequence absent in mRNA	Kozak sequence present in mRNA which is located few nucleotides upstream of start site (Kozak sequence assists initiation process of translation)
Initiation codon is usually AUG (occasionally GUG or UUG)	Initiation codon is usually AUG (occasionally GUG or CUG)
Smaller subunit of ribosome (30S) recognizes the SD sequence in the mRNA during translation initiation	Smaller subunit of ribosome (40S) recognizes the 5'cap of mRNA during initiation
First tRNA is special type namely Met-tRNA$_f$	First tRNA is Met-tRNA
Formylation of first amino acid in the protein synthesis (methionine)	No formylation of methionine, first amino acid in the protein synthesis

Adapted from "*Eukaryotic DNA Replication*". (n.d.). BRAINKART. Retrieved October 11, 2023 from https://www.brainkart.com/article/Eukaryotic-DNA-Replication_27554/; Lakna (September 8 2019). "*Difference Between Prokaryotic and Eukaryotic RNA Polymerase*". PEDIAA. Retrieved October 11, 2023 from https://pediaa.com/difference-between-prokaryoticand-eukaryotic-rna-polymerase/.

Microalgae show high potential for industrial biotechnology not only because they are photosynthetic, but also because of the bioactive compounds they produce. The chlorophyte *Chlamydomonas reinhardtii* has been used to develop molecular tools that permit genome engineering. In particular, the chloroplast is a compartment that offers many advantages. It has a small genome, with no more than a hundred genes, and homologous recombination allows targeted integration of foreign information. Several reports have documented the successful expression of recombinant proteins from the plastome of *C. reinhardtii*. The goal of this manuscript is to outline the main features that make this organelle a convenient platform for the production of valuable proteins, and along the way to review its origins and adaptations not only in *C. reinhardtii* but also in other algae.

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ISBN: 978-9978-25-250-5

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