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Investigations of Chlamydomonas reinhardtii ergosterol biosynthesis

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INVESTIGATIONS OF CHLAMYDOMONAS REINHARDTII ERGOSTEROL BIOSYNTHESIS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Department of Biological Sciences

by

Kristy Marie Brumfield
B. S., Xavier University of Louisiana, 2003
December 2009
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<tr>
<td>BLAST</td>
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</tr>
<tr>
<td>CAS</td>
<td>cycloartenol synthase</td>
</tr>
<tr>
<td>CDI</td>
<td>Δ8-Δ7 sterol isomerase</td>
</tr>
<tr>
<td>CPI</td>
<td>cyclopropyl sterol isomerase</td>
</tr>
<tr>
<td>DDY</td>
<td>David Donze Yeast strain</td>
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<tr>
<td>DOXP</td>
<td>1-deoxy-D-xyulose-5-phosphate</td>
</tr>
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<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>JGI</td>
<td>joint genome institute</td>
</tr>
<tr>
<td>IFT</td>
<td>intraflagellar transport</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl diphosphate</td>
</tr>
<tr>
<td>LAS</td>
<td>lanosterol synthase</td>
</tr>
<tr>
<td>MVA</td>
<td>mevalonic acid</td>
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<tr>
<td>Q-RT PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>SMT</td>
<td>sterol methyl transferase</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
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ABSTRACT

Ergosterol is the major sterol found in the membranes of *Chlamydomonas reinhardtii*. While past studies have identified some ergosterol mutants in *C. reinhardtii*, very little is known about sterol biosynthesis pathways in this species. With the elucidation of the *Chlamydomonas* genome, bioinformatics analysis has allowed us to determine potential genes involved in ergosterol biosynthesis. With this knowledge, a working model of the pathway was designed for future analysis. Several of the ergosterol biosynthetic genes were analyzed in respect to their role and involvement in flagellar regeneration. These genes were upregulated during the regrowth of the flagella. Also *Chlamydomonas* strains lacking flagella were analyzed by Q-RT PCR to determine what role ergosterol biosynthetic genes played in the absence of their flagella. Finally, one of the genes with homology to the yeast sterol C-5 desaturase, *ERG3*, was chosen for further analysis. To test whether *ERG3* of *C. reinhardtii* had a similar function, yeast *Saccharomyces cerevisiae ERG3* knockout strains were created to assess whether a plasmid expressing the *Chlamydomonas ERG3* could complement the deletion. These *erg3* null mutants were transformed with a vector expressing *ERG3* cDNA from *C. reinhardtii* driven by the yeast *ADH1* promoter, and this expression was able to restore ergosterol biosynthesis and reverse phenotypes associated with lack of *ERG3* function. Complementation of these *erg3* null phenotypes strongly suggests that *ERG3* in *C. reinhardtii* functions as a sterol C-5 desaturase. Results from this dissertation provides the groundwork for future experimentation in the field of sterol lipid research in *Chlamydomonas reinhardtii.*
CHAPTER 1
REVIEW OF LITERATURE

1.1 Overview of Sterol Lipids

Sterols are lipid-soluble molecules containing a four-ring structure with a 3β-
hydroxyl group and an aliphatic side chain of 8 or more carbon atoms. Sterols fall under
the macromolecule class of lipids. They are resistant to saponification, and are found in
abundant quantities in all animal and plant tissues. The physiology and viability of
eukaryotic cells depends on the presence or absence of sterols. Therefore, there is great
variation between the classes of sterols in specific cell types as well as species. The
major differences in sterols include the number, location and orientation of double bonds
and side chains (Fahy et al., 2005). The nomenclature of sterols is basically defined and
based upon the “tetracyclic skeleton” as seen in Figure 1.1 (Moore, 1993). The
tetracyclic skeleton is composed of carbons 1-27 while carbons 28, 29, 30, 31, and 32, are
subsequently added to the molecule (Fahy et al., 2005). In the sterol nucleus, the up
conformation is referred to as β while the down conformation is α. The conformational
orientation of the side chain is the exact opposite, as the up conformation is denoted as α,
while the down conformation is described as β (Fahy et al., 2005). For example, the
major differences associated with algal, plant and fungal sterol lipids have a great deal to
do with variations in the side chains (Moore, 1993). Lanosterol, an ergosterol precursor
in yeast produces sterols in the S configuration, while in plants, cycloartenol is the
precursor, and its sterol products are found in the R configuration (Moore, 1993).
Understanding this complex nomenclature system is integral for proper identification of
sterol lipids in Chlamydomonas reinhardtii.
Figure 1.1 Sterol nomenclature. Each ring is denoted as either A, B, C, or D. \( \Delta \) with a superscript number denotes the location of a double bond from that carbon to the carbon with the next higher number. Double bonds between carbons can also be indicated by the carbon with the lower number followed by the carbon with the higher number, the latter in parentheses. The position of a carbon in the molecule is indicated by C- followed by a number. C with a subscript number explains the total number of carbons in the molecule. Picture adopted from reference (Moore, 1993).
While naming sterol lipids and understanding their phylogenetic differences have been very important aspects of lipid biochemistry, the function of lipids and sterols has been an even more intriguing field of study. Lipids are a major component of cell membranes. Membranes are dynamically changing entities that possess not only lipids but proteins as well. The Singer-Nicholson fluid mosaic membrane model proposed that there are random components of the membrane that allow for indiscriminate rotational freedom of molecules (Singer, 1972, Singer & Nicolson, 1972). After years of research, scientists have been able to refine this model. It is now thought that this random movement of molecules is in actuality very unambiguous. Scientists argue that there needs to be a paradigm shift from the previous Singer-Nicholson model to a more updated model incorporating membrane microdomains. There are six characteristics of cell membranes that do not fit with the Singer-Nicholson model: (i) specific patterns of distribution for receptors in the plasma membrane, (ii) lipid associations with signaling molecules, (iii) unrestricted diffusion of particles through a shorter diffusion barrier, (iv) lipids associate with specific membrane proteins, (v) presence of integral proteins maintain membrane domains, (vi) protein reorganization to allow for protein-protein and lipid-protein interactions (Vereb et al., 2003). These physical characteristics of the membrane enhance the previous model of a more random unorganized, description of lipids and proteins as described by Singer and Nicholson. The movement of lipids and proteins in the membrane are far from unrestricted.

Membranes are essential components of eukaryotic cell function. The plasma membrane is particularly significant since it separates the inner compartments of the cell from the outside environment. Sterols are important lipid components of the plasma membrane, and have been shown to play crucial roles in several cellular functions.
Detailed studies of sterols have focused primarily on understanding how sterols influence membrane fluidity and permeability. The effects of sterols on the activity of membrane bound enzymes and rates of cell growth have also been of significant interest.

1.2 Physiological Role of Sterols

As vital components of the plasma membrane, sterols play a critical role in maintaining the structure and viability of the cell. The primary biophysical role of sterols is to maintain the spacing between phospholipid molecules of the membrane bilayer in order to restrict van der Waals forces (Figure 1.2). Homeostasis is best maintained in the cell when the membrane is functioning at an optimal fluidity. At high temperatures, lipids can weaken and become too fluid-like, while at lower temperatures, the lipid bilayer can become too rigid and solidify. The presence of sterols prevents solidification at lower temperatures and melting or weakening of the membrane at higher temperatures. While this is not the only physiological role of sterols, this phenomenon has been quite intriguing to the scientific community. Maintenance of the membrane fluidity continues to be a well-defined role of sterols (Solomon et al., 2008).

One prominent sterol found in the membranes of many fungal species is ergosterol, which is the primary sterol lipid in Saccharomyces cerevisiae (Daum et al., 1998). The post-squalene biosynthesis of ergosterol in yeast has been well defined (Arthington-Skaggs et al., 1996, Osumi et al., 1978). Enzymes of the ergosterol biosynthetic pathway have been found to be major targets for drug interactions (Balliano et al., 1989, Rao & Grollman, 1967, Siegel & Sisler, 1965, Cohen & Eaton, 1979), as many modern antifungal drugs on the market were derived specifically to target ergosterol biosynthesis (Cowen & Steinbach, 2008, White et al., 1998). For example, azole drugs directly affect ERG11, a cytochrome p450 family protein, which is
Figure 1.2  Schematic Diagram of the Phospholipid Bilayer. (A) Phospholipid bilayer with an integral membrane protein. (B) Phospholipid bilayer with an integral membrane protein embedded with sterol molecules to increase the spacing and maintain fluidity of the bilayer. This figure was adapted from the original Singer-Nicholson model (Singer, 1972, Singer & Nicolson, 1972).
responsible for converting lanosterol to 4,4'-dimethyl cholesta-8,14,24-triene-3-beta-ol (Aoyama et al., 1981, Parks et al., 1995, Kalb et al., 1987, Barrett-Bee & Dixon, 1995).

Overall, ergosterol plays a very important role in other lipid biosynthetic pathways. Several processes in phospholipid biosynthesis are regulated by ergosterol. Phospholipid composition is affected by the transfer rate of phospholipids by phosphotidylinositol-transfer protein when stimulated by ergosterol (Szolderits et al., 1989). Ergosterol is known to upregulate the activity of phosphatidylinositol kinase, (Dahl et al., 1987, Dahl & Dahl, 1985) and the methylation of phosphatidylethanolamine to phosphatidylcholine is also stimulated by ergosterol (Kawasaki et al., 1985). In addition, evidence exists for crosstalk between ergosterol and other lipid metabolic pathways. Ceramide production is down-regulated when 3-hydroxy-3-methylgutaryl-CoA reductase, a critical enzyme in ergosterol biosynthesis, is inhibited by lovastatin in mammalian cells (Storey et al., 1998). Microarray analysis has shown that expression of genes involved in long chain fatty acid synthesis is significantly affected when ergosterol biosynthesis is blocked (Bammert & Fostel, 2000).

Specific lipids are responsible for increasing the order and organization of membrane domains. These unique proteins and lipids organize to form lipid rafts. In vertebrates, three specific lipids act as major precursors to the lipid raft: sphingolipids, phospholipids and cholesterol, the structures of which are depicted in Figure 1.3. Lipid rafts have roles in cell signaling, protein synthesis, cell cycle regulation, and phosphorylation of different regulatory kinases (Simons & Toomre, 2000). The outer leaflet of rafts is normally comprised of glycosphingolipids and sphingomyelin, while the inner leaflet contains glycerolipids with fatty acyl chains. In vertebrates, cholesterol is a major component of the lipid rafts (Brown & London, 1998). Cholesterol is extremely
Figure 1.3 Three major molecules involved in lipid raft formation. (A) cholesterol; (B) phospholipid; (C) sphingolipid.
rigid due to its four ring structure, and thereby functions to increase the fluidity of the membrane by thermodynamically reacting with other lipids to change the gel phase to a liquid ordered phase. With cholesterol present, the membrane is inhibited from moving into the crystalline state. Cholesterol buries itself between the hydrocarbon tails of the sphingolipids, and in its absence lipid rafts lose all integrity and may fall apart (Simons & Toomre, 2000). The Golgi apparatus is thought to be the organelle in cells in which lipid rafts are assembled (Brown & London, 1998). After synthesis of cholesterol and sphingolipids in the endoplasmic reticulum these molecules are then moved to the Golgi for lipid raft assembly (van Meer, 1989). In recent years, researchers have been able to identify the lifetime of lipid rafts in the cell. It was previously thought that these microdomains were very stable, but now information has become available indicating that lipid rafts are short-lived in the cell, perhaps existing for only “tens of seconds” (Edidin, 2001). In general, rafts are relatively small in size in order to maintain “raft-borne signaling proteins” in a state of inactivity (Simons & Ehehalt, 2002). When activated, rafts assemble into larger platforms by which functionally related proteins can then interact and crosstalk (Simons & Ehehalt, 2002). There are several diseases that are related to defects in assembly of lipid rafts. Those include Alzheimer’s disease, Parkinson’s disease, diabetes, and osteoarthritis (Simons & Ehehalt, 2002). Lipid rafts are extremely important for maintaining the viability of an organism. It is thought that changing cholesterol levels in the cell can affect the “association pattern” of lipid rafts (Matko & Szollosi, 2002). Cellular uptake of cholesterol can affect transcriptional regulation of genes involved in the pathway as well as the ability to properly cluster and assemble rafts (Tall et al., 2002).
1.3 Overview of Sterol Biosynthesis

Two major pathways for sterol biosynthesis have been found in eukaryotes. Fungi and vertebrates synthesize sterols with lanosterol as an intermediate, while plants synthesize sterols using cycloartenol as an intermediate. In these pathways the biosynthetic steps from isopentanyl PP (IPP) to squalene epoxide are the same. However, the cyclization of squalene epoxide is where the two pathways diverge producing either cycloartenol or lanosterol. (Figure 1.4). Less is known about the biosynthesis of sterols in algal species. Red algae, green algae and diatoms are thought to make cycloartenol while dinoflagellates have been reported to make lanosterol (Baker & Kerr, 1993, Giner, 1993).

There is a some evolutionary difference between sterols found in mammals, fungi, higher plants, algae, and some protozoans (Bloch, 1983). There are significant structural differences between cholesterol and ergosterol, the central sterols of eukaryotic organisms (Figure 1.5). Ergosterol has two double bonds at C-7 and C-22 and a side chain methyl group at C-24. It has been suggested that cholesterol is found in mammalian cells because of its ability to function in the optimization of membrane lipid packing for the purpose of maintaining rigidity and fluidity dependent on temperature and cell size (Bloch, 1983, Arora et al., 2004), while ergosterol is favored in lower eukaryotic species to enhance membrane disorder as a substitute for the production of unsaturated fatty acids (Bloch, 1983).

1.4 Sterols in Eukaryotic Organisms

Sterols are found in the membranes of most eukaryotic organisms (Bloch, 1983). Even though there are similarities, the differences between mammalian, fungal and plant sterols are quite distinct. The major sterol found in mammalian cell plasma membranes
Figure 1.4 Schematic diagram showing the evolutionary branch from IPP to lanosterol and cycloartenol.
Figure 1.5 Diagrams of the structures of cholesterol and ergosterol.
is cholesterol, which is an intermediate in the synthesis of Vitamin D, bile salts and hormones. Plant cells contain sitosterol, while fungal cell membranes have ergosterol as the major sterol components (Bloch, 1983). Plants have a variety of sterols including sitosterol, 24-methyl cholesterol and stigmasterol (Benveniste, 2004). Ergosterol is also found in the membranes of protozoan parasites, Leishmania and Trypanosoma (Mbongo et al., 1998), and interestingly, the eukaryotic model system, Drosophila melanogaster, where it is a major sterol lipid (Rietveld et al., 1999).

While the study of sterol biosynthesis in eukaryotic organisms has looked primarily at the functional role of sterols in terms of end-product sterol formation, scientists have also examined the production of sterols not as strict precursors but also as hormonal regulators as well (Norton & Nes, 1991). In mammalian cells, cholesterol serves as the precursor to the hormonal regulators, testosterone and estrogen. Like testosterone and estrogen, brassinosteroids in higher plants act as sterol hormones to coordinate several cellular processes (Bajguz, 2007). To date, no steroidal hormones have been identified in C. reinhardtii. However, in another algal species, Prototheca wickerhamii, two new steroidal triene hormones have been identified (Norton & Nes, 1991). Interestingly, a great deal of research has also been done to elucidate specific steps in ergosterol biosynthesis by which pathogenic organisms like Trypanosoma brucei might be eradicated. Scientists have shown that the import of cholesterol in this model system does not diminish ergosterol biosynthesis or the hormonal importance of the molecule in T. brucei, but introducing an analog of the sterol C-24 methyltransferase does effectively stop the growth of the organism in the bloodstream (Zhou et al., 2007). This is just one example of how sterols play multiple roles in the cell across several different eukaryotic species, and how an understanding of their synthetic pathways may
be exploited for therapeutic purposes.

1.5 Isoprenoid Pathway

Isoprenoid synthesis has been studied since the late 1950s and has given great insight into the biochemical process in photosynthetic higher plants and algae. Sterols are a direct end product of isoprenoid biosynthesis. For many years there has been a debate about whether or not there are two distinct pathways for the synthesis of isopentenyl diphosphate (IPP). It is thought that IPP serves as the common C₅ precursor that aids in the production of most isoprenoids including sterols.

\[
\begin{align*}
3 \text{ Acetyl-CoA} & \rightarrow \text{Mevalonate} & \rightarrow \text{IPP} \\
\text{OR} \\
\text{Pyruvate} & \rightarrow \text{1-deoxy-d-xylulose-5-phosphate} & \rightarrow \text{IPP} \\
\text{GAP} &
\end{align*}
\]

**Figure 1.6** The classical formation of Δ³-isopentenyl diphosphate (IPP) by the acetate/mevalonate pathway and the alternative 1-deoxy-d-xylulose-5-phosphate (DOXP) pathway. *Chlamydomonas reinhardtii* uses the DOXP pathway to synthesize sterols in the cell.

By radioactive incorporation experiments, it has been shown that *C. reinhardtii*, uses a mevalonate independent pathway to synthesize its sterol molecules (Lichtenthaler, 1999). IPP is generated by the DOXP pathway as seen in Figure 1.6 (Schwender *et al.*, 1999). By labeling 1-deoxy-d-xylulose-5-phosphate, it has been discovered that incorporation of the molecule was found in the isoprene units of *C. reinhardtii* (Steele *et al.*, 1969).

From an evolutionary perspective, the DOXP pathway is distributed among
photosynthetic algae and higher plants. *Euglena* appear to have lost the DOXP pathway for the synthesis of IPP (Lichtenthaler, 1999, Van Den Hoek, 1997). They rely solely on the mevalonic acid pathway for the production of isoprenoid products, whereas green algae such as *C. reinhardtii* have been able to utilize the DOXP pathway for sterol synthesis (Steele *et al*., 1969). It is also maintained that in *Arabidopsis* and *C. reinhardtii*, DOXP synthase possesses a signal peptide sequence implying its localization for the pathway in the plastids (Mandel *et al*., 1996). Isoprenoid synthesis has been known to take place in the chloroplasts (Lichtenthaler *et al*., 1997, Moore, 1993).

*Cyanobacteria* use the DOXP pathway and are thought to be the “progenitors of the chloroplast,” but through the co-evolutionary process with eukaryotes, the DOXP process was compartmentalized in higher plants (Mandel *et al*., 1996, Nicolson & Singer, 1972).

In conclusion, the early studies in isoprenoid biosynthesis demonstrated the diverse nature of algal, fungal and higher plant metabolic pathways. *Chlamydomonas reinhardtii* utilizes the DOXP pathway for the production of isoprenoids. Future work in this area will be of utmost importance for characterizing the genes and enzymes that coordinate the synthesis of isoprenoids and precursors to sterols in the membrane of this model system.

1.6 *S. cerevisiae* as a Model Organism for Study of Ergosterol Biosynthesis

Much like cholesterol biosynthesis, ergosterol synthesis has been widely studied. The yeast, *Sacchromyces cerevisiae*, has been used as a model system for the study of this membrane component. Many antifungal agents have been created to target the enzymes of ergosterol synthesis, thus killing the fungal organism. The ergosterol pathway is catalyzed by at least eleven enzymes (Figure 1.7). These enzymes perform a series of biochemical steps to change the unsaturated, linear squalene structure to the four
ring sterol, ergosterol. The chemical name for ergosterol is 24\(\beta\)-methylcholesta-5,7,22-triene- 3\(\beta\)-ol. Scientists are able to grow cultures of \textit{S. cerevisiae} at a relatively low cost and manipulate genes of the ergosterol biosynthetic pathway in order to better identify the function of each enzyme (Daum \textit{et al.}, 1998, Parks & Casey, 1995). There is a yeast knockout library that is commercially available. Ergosterol biosynthetic genes have been deleted and their phenotypes are well catalogued and documented (http://www.yeastgenome.org). As with \textit{C. reinhardtii}, the entire genome of \textit{S. cerevisiae} is sequenced and available for analysis. The yeast genome contains a total chromosomal sequence of approximately 12 Mb with a haploid set of 16 chromosomes that range in sizes as small as 200 kb to 2,200 kb (Sherman \textit{et al.}, 2002). The sequence, released in 1996, identified 6,183 possible ORFs encoding proteins greater than 100 amino acid long (Sherman \textit{et al.}, 2002). Functional complementation of ergosterol biosynthetic genes in yeast can be performed with great efficiency (Rothstein, 1983, Parks \textit{et al.}, 1999). In addition, yeast also serves as a great model system for other molecular biology experimental approaches such as two hybrid analysis and serial analysis of gene expression (SAGE) (Sherman \textit{et al.}, 2002).

1.7 \textit{C. reinhardtii} as a Model Organism for Study of Ergosterol Biosynthesis

\textit{C. reinhardtii} has been well defined as a model organism for biochemical and molecular studies. Historically, the organism has been manipulated for analysis of photosynthesis, light perception, cell cycle control, flagellar and basal body structure and function, as well as chloroplast biogenesis (Harris, 2001, Rochaix, 1995). The organism is both unicellular and eukaryotic, and it can be grown photosynthetically, or in the presence of a carbon source such as acetate. There are two mating types (\textit{mt+} and \textit{mt-})
Figure 1.7 The Ergosterol Pathway in *S. cerevisiae*. The schematic diagram shows the reactants, enzymes and precursors to ergosterol in yeast.
that can be easily crossed for genetic studies. The nuclear, chloroplast and mitochondrial genomes have well defined markers for efficient transformation and genetic manipulation (Boynton et al., 1988, Kindle, 1990, Dunahay, 1993, Shimogawara et al., 1998). Mutant stocks and DNA clones are readily available for analysis (http://www.yale.edu/rosenbaum/ chlamy_resources.html). EST clones and genomic sequences are released and updated on a one to two year basis. There have been four versions of the Chlamydomonas genome released (http://www.biology.duke.edu/chlamy_genome/index.html, http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). The genome size is estimated to be approximately 1x10^8 base pairs (Harris, 2001, Rochaix, 1995). There is a codon bias that favors a high GC content, and upstream of these GC rich sequences there are adenosine rich elements (Rochaix, 1995). On average each gene contains approximately four intron regions of 219 bases within a kilobase of coding sequence, allowing the number of introns to be approximately equal to that of the coding region (Lefebvre & Silflow, 1999).

### 1.8 Sterol Biosynthesis in *C. reinhardtii*

*Chlamydomonas reinhardtii* is a unicellular alga that has great potential as a model system for lipid and membrane studies (Harris, 2001). Lipids play significant roles in the integrity and functioning of the plasma membrane as well as organelle membranes. Sterols are a major component of many membranes, including those found in algae.

Flagellar membranes have been studied extensively. In *Chlamydomonas*, the flagellar membrane seems to be somewhat different from that of the whole cell. Prior research has shown that the flagellar membrane is not a continuation of the plasma
membrane, but is quite unique in its components (Gealt et al., 1981). Gealt showed that in whole cell extracts 14:0, 16:0, 16:2, 16:3, 18:0, 18:1, 18:2 and 18:3 fatty acids, while flagella extracts only contained 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids. He also showed that ergosterol and 7-dehydroporiferasterol were the two major sterols of the flagellar membranes in C. reinhardtii. The sterol composition of the membrane was reported to be 55% ergosterol while 45% was 7-dehydroporiferasterol (Gealt et al., 1981). To further study the function of sterols in this model system, Janero et al. looked at variations in sterol synthesis during different phases of the cell cycle (Janero et al., 1982). He reported high rates of sterol synthesis in mid to late G1 phase of the cell cycle or the light period, while it drops dramatically during the dark period.

In addition to lipids, proteins also are a major component of the membranes. Several proteins are contained in the two posterior flagella of C. reinhardtii, but one major protein is thought to be dominant in the flagellar membrane (Nakamura et al., 1996, Snell, 1976). This mastigoneme protein (Figure 1.8) extends from the outer membrane of the flagella into the surrounding medium. These proteins function to increase swimming velocity as well as serving to anchor the cells together during the mating process (Witman et al., 1972). The mechanism by which this protein reaches the tip of the flagella has not been elucidated.

Reflagellation is a naturally occurring phenomena in C. reinhardtii, allowing the flagella to regenerate after excision or pH shock (Harris, 2001). Intraflagellar transport (IFT) is a microtubule-dependent motility process that was first observed in the lab of Joel Rosenbaum at Yale University by Keith Kozminsksi (Kozminsksi et al., 1993, Sloboda, 2002). This process occurs in an anterograde fashion by which soluble protein particles are moved from the cell body to the tip of the flagellum or cilium and back to
the cell body (Rosenbaum & Witman, 2002). IFT rafts, unlike lipid rafts, do not contain lipids but do occur within the cilia or flagella beneath the plasma membrane (Sloboda, 2002, Silflow & Lefebvre, 2001).

Figure 1.8 TEM pictures of isolated flagella with the intact membrane after pH shock. Mastigonemes are the smaller structures extending off of the sides of the flagella.


1.9 Goal of This Dissertation

The goal of this dissertation is to elucidate sterol biosynthesis in *C. reinhardtii*. While, sterol biosynthesis in *Chlamydomonas* has not been studied much since the late
1990s, I truly hope that by characterizing the genes of the biosynthetic pathway and providing experimental data supporting evidence of this pathway, the scientific community will be excited about new pursuits in the area of lipids research. Ergosterol biosynthesis overlaps with many metabolic pathways, and it is of particular interest to also show interactions between ergosterol biosynthesis and reflagellation. The overall objectives of this work are the following: (1) to identify these biosynthetic genes for future studies of biodiesel alternative fuel production and (2) find the link between sterols and the ability to regenerate flagella and cilia.
CHAPTER 2

MATERIALS AND METHODS

2.1 *S. cerevisiae* Strains and Growth

Yeast strains were constructed by homologous recombination using primers 5’-TGC ATT TGT AAA AAA AGA TAA AA GAA AAA TAT TCG TCT AGA TTT GAG ATG CAG ATT GTA CTG AGA GTG C-3’ (forward) and 5’-TCT TGA ACG TGA AAG AAA GAA AAA AGA TGA GAC AAA CAA GGC AAC CGT ATC TCC TTA CGC ATC TGT GCG G-3’ (reverse) to amplify the yeast *URA3* gene flanked by *ERG3* sequence. Deletion of *ERG3* was done in a diploid strain DDY2 (a diploid version of *S. cerevisiae* W303), which was then sporulated to produce haploid cells (see below). Yeast were cultured in YPD (1% yeast extract, 2% peptone, 2% dextrose) or YMD (synthetic minimal medium, 2% dextrose, U. S. Biologicals catalog # Y2025) supplemented with required amino acids and nucleotides (Sherman, 1991). Unless otherwise stated, all yeast operations were carried out according to standard procedures (Guthrie & Fink, 1991). To construct an *ADH1* promoter vector, the plasmid pOAD used (Dr. Stan Fields, University of Washington) was modified as follows. The *GAL4* coding sequence was removed by digestion with *Hind* III, and re-ligated to produce the *ADH1* promoter control vector pDD1193, and *ERG3* genes were cloned into the resulting unique *Hind* III site. Expression of *S. cerevisiae* and *C. reinhardtii* *ERG3* cDNA from the *ADH1* promoter of yeast expression vector pDD1193 was carried out by culture of the transformed yeast strain in YMD lacking leucine.
2.2 Yeast Competent Cell Preparation and Transformation

Single yeast colonies were inoculated and grown overnight. The following the morning, cultures were diluted to an optical density of 0.2 (A$_{600}$), and cells were grown until reaching an approximate optical density of 0.7. Cells were harvested by centrifugation (2000xg for 5 minutes) at room temperature, and the cell pellet was resuspended in 1 mL of 1xTEL (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM LiAc) per 10 mL culture volume and rocked overnight at room temperature. Yeast were transformed with plasmids using the protocol of Geitz et al (Gietz & Woods, 2002).

2.3 Yeast Genomic DNA Extraction

Yeast genomic DNA was extracted by the Winston protocol (Hoffman & Winston, 1987).

2.4 Tetrad Dissection

Heterozygous diploid cells were cultured on YPD plates, then transferred to spore plates. A 1mm mass of cells was resuspended in 6µL Zymolase (1mg/mL) for each sporulation and incubated for 2 minutes at room temperature. After incubation, 300µL of water was added to each sample. Each sample solution was then spread along the upper portion of a petri dish and tetrads were separated with an Olympus B201 Dissecting scope. Each plate was incubated at 30º C, and cells were re-patched to a master plate. Each master plate was then replica plated to YMD dropout plates and mating tester lawns to determine genotype.

2.5 C. reinhardtii Strains and Growth

Dr. R. K. Togasaki of Indiana University, Bloomington provided Wild type C. reinhardtii 137+. Rogene Schnell, University of Arkansas-Little Rock, provided strain D66 (nit2, cw15, mt$^+$). Strains cc477, cc478, cc479, cc480, cc482 and cc652 were ordered from the Chlamydomonas Culture Collection. Cultures were grown on Tris Acetate
Phosphate (Sueoka, 1960) (TAP) medium plates. They were inoculated into 100 mL of TAP medium (Sueoka, 1960) and set to grow with continuous light (300 µE m\(^{-2}\)S\(^{-1}\)) and shaking for 2-3 days. Aliquots of the culture were transferred to a larger flask (Sueoka, 1960) or carboy and bubbled with high CO\(_2\) (5% CO\(_2\) in air) for several days. Cells were then collected for further analysis: pH shock or RNA preparation.

### 2.6 Chlamydomonas Core Library

The *Chlamydomonas* cDNA core library was purchased from the Chlamydomonas Center (http://www.chlamy.org/). The library was amplified using the host strain XL1 Blue MRF’ (Stratagene catalog number 200301) according to the manufacturer’s instructions at http://www.stratagene.com/lit/manuals/aspx (catalog number 236201).

### 2.7 cDNA Cloning

The *S. cerevisiae ERG3* protein coding sequence (accession number NP_013157) was used to identify the *C. reinhardtii* EST with the best homology using the BLAST server (http://genomeportal.jgi-psf.org/Chlre4/Chlre4.home.html). Primers were designed based on the EST sequence in order to clone the coding region of cDNA from the core library described above. The primers used to clone the cDNA by PCR included the forward primer 5′-GCG GCC GCC GAT CGA AGC TTA ATG TCA ACC TCG CTC AAA ATG A-3′ and the reverse primer 5′-GCG GCC GCC GAT CGA AGC TTT ACT GCG CCT TGA CGG CCT T -3′.

### 2.8 Bioinformatics and Sequence Analysis

Information about genes found in *Chlamydomonas* was gathered from searching the genomic database, which yielded gene models, scaffold and chromosome locations (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). Ergosterol biosynthetic enzymes
were obtained from *Saccharomyces cerevisiae* sequences on the NCBI protein database. The *C. reinhardtii* EST database using the BLAST program provided alignments with the yeast amino acid sequences (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1997). In order to determine the correct gene, E-values were considered. The gene with the smallest E-value assignment was chosen as the best choice for the gene. E-values are often used to differentiate between multiple copies of the same gene in the genome. The E-value is a statistical value that estimates the probability of similarity between a given sequence and the database of sequences. Splice sites of the exon/intron and open reading frames were identified by the method described by Silflow in 1995 (Silflow *et al.*, 1995). ChlorP, TargetP, and SortP were used in generating data for protein prediction. These programs included hyperlinks to the Expasy server (http://ca.expasy.org/tools/#translate). The Pfam database was used to identify possible domains in the protein sequences (http://pfam.sanger.ac.uk). ClustalW sequence alignment provided similarities in protein sequence (http://www.ebi.ac.uk/Tools/clustalw2/). PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) was used to predict the localization of specific proteins (http://psort.nibb.ac.jp/). The *Saccharomyces* genome database provided information about the function of the enzymes in yeast (http://www.yeastgenome.org/). Multiple sequence alignment programs from the European Bioinformatics Institute EMBL-EBI server (http://www.ebi.ac.uk/clustalw/) were used for sequence analysis and alignment data. The Prosite database from EBI server (http://ca.expasy.org/prosite/) was used to identify putative consensus motifs for specific domains. The *C. reinhardtii* genomic database provided information about the genomic sequence, intron-exon structure, as well as information regarding potential membrane spanning regions of the protein coding sequence (http://genomeportal.jgi-
2.9 Drug Resistance Screen

Cycloheximide was purchased from Sigma Aldrich (catalog# C-7698-1G). *S. cerevisiae* minimal medium was prepared as previously described with the addition of cycloheximide (Dudley *et al.*, 2005) at a final concentration of 0.13 µg/mL from a stock solution of 2mg/ml in 100% ethanol.

2.10 Lipid Extraction

Yeast cells were grown to an optical density at A<sub>600</sub> of 0.7-0.8. The cells were precipitated at 2000 rpm for 5 minutes and resuspended in 4mL hot isopropanol (70º C) for 30 minutes. The cell wall was disrupted by vortexing with 0.5mm glass beads for 2 minutes. Cell pellets were then resuspended in 4mL hot isopropanol and lipids were extracted at 70º C for 2 hours. Next, 4mL of chloroform/methanol (1:2) and 2mL of 1 M KCL were added to the lipid extracts, which were vortexed and centrifuged at 5000 rpm on a benchtop centrifuge for 5 minutes. The top aqueous phase was discarded and the previous extraction step was repeated three times. Then, 2mL of water were then added to the samples. The samples were vortexed and centrifuged at 5000 rpm for 5 minutes, and the aqueous phase was discarded again. Lipids were dried under nitrogen and stored at -80º C. The lipid extraction protocol was derived from the Bligh and Dyer method (Bligh & Dyer, 1959).

2.11 GC/MS Analysis

Ergosterol standard was purchased from Fluka. N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was used as the silylating reagent and was purchased from Sigma Aldrich (catalog #33084). 100µL of BSTFA was added to dried lipid samples and heated to 60º C for 30 minutes. Derivatized samples were resuspended in 25µL of
dichloromethane and prepared for analysis. GC/MS was carried out on an Agilent 6890 gas chromatograph with an autosampler and Agilent 5973 mass selective detector. Then, 1µL of sample was injected into a splitless system with a flow rate of 1mL/ min for the carrier gas, helium. The front inlet temperature was maintained at 250º C, while the mass selective detector line heater was at 280º C. The oven temperature was programmed to a final temperature of 300º C with an initial temperature of 100º C. The GC column, DB-5ms, was 30 meters by 250 µm of internal diameter with a film thickness of 0.25 µm. Data were acquired by selective ion monitoring to detect ions at m/z 143, 211,337, 363, 468.

2.12 Flagellar pH Shock

*Chlamydomonas reinhardtii* wt 137 mt- cultures were grown on Tris-Acetate Phosphate (TAP) agar plates, and aseptically inoculated after 5 days into Tris-Acetate Phosphate media for 2 days with continuous shaking under light. TAP cultures were transferred to Minimal media (MIN) and incubated at 30ºC for 48 hours under continuous light and CO₂ with continuous shaking. Cultures were harvested by centrifugation in a Beckman J2-21 centrifuge at 4ºC, 480g for 10 minutes in a JA-10 rotor. The original MIN medium was saved for resuspension of cells prior to flagellar regeneration analysis. The cell pellet was resuspended as a control in 30mL of the original MIN medium and incubated at 30ºC on a shaker under light.

Acid shock for removal of flagella: The pellet was resuspended in 50mL of 10mM Tris-HCl buffer, pH 7.8 + 7% sucrose (w/v) at 4ºC. Cells were deflagellated by addition of 0.5N acetic acid to pH 4.5 and held at acid pH for 45 seconds, following which the pH was raised to 7.8 with 0.5N KOH (10). 15mL aliquots of the shocked cells were under-layered with 20mL aliquots of 10mM Tris-HCl buffer, pH 7.8 at 4ºC + 25%
sucrose (w/v) and centrifuged in a Beckman J2-21 centrifuge at 4°C with a JA-20 rotor for 11 minutes at 2000g. The 7% sucrose layer and interface was removed and flagella were purified by centrifugation at 27,000g.

2.13 Total RNA Isolation

Trizol reagent (Invitrogen, Carlsbad, CA) was used to extract RNA from C. reinhardtii. Cells were harvested and transferred to 50 ml conical centrifuge tubes. Cells were then spun at 3000 rpm for 5 minutes at a final temperature of 4°C. The pellet was resuspended and transferred to 1.5 mL Eppendorf tube with one mL of Trizol. Cells were then vortexed and left to incubate for one hour at room temperature. After the duration of the hour 200 µL of chloroform was added. To mix the sample, the tubes were again vortexed and incubated at room temperature for 5 minutes. The samples were centrifuged at 10,000 rpm for 15 minutes at 4°C.

The aqueous phase of approximately 600 µL was removed and transferred into a fresh 1.5 mL Eppendorf tube. 0.5 mL of isopropanol was added and the samples were again incubated for 30 minutes. The samples were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was removed, and the RNA pellet was washed with 1.0 mL 75% ethanol and then centrifuged at 8,000 rpm for 5 min. The supernatant was removed and the pellet was air dried for an hour. The RNA was finally treated with Ambion Turbo Dnase. The manufacturer’s protocol was followed and the samples were then treated by an RNA clean-up using Qiagen’s RNeasy Clean-up Kit. Total RNA concentration was calculated by absorbance at 260 nm, using the conversion equation: 1 OD$_{260}$ = 44 µg of RNA•mL$^{-1}$. 

27
2.14 Quantitative RT (Real Time) PCR

3μg of total RNA was used as template for synthesis of cDNA. Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) was used to synthesize the cDNA according to manufacturer’s instruction. An aliquot of one-tenth synthesized cDNA was used as the template with Takara Bio Sybr Green Premix Ex Mix (Takara-Bio, Madison, WI) for quantitative PCR in an ABI Prism 7000 sequence detection system following the manufacturer’s instructions (Applied Biosystems, Foster City, CA).
CHAPTER 3

BIOINFORMATIC ANALYSIS OF ERGOSTEROL BIOSYNTHESIS
IN C. REINHARDTII

3.1 Introduction

In the new age of bioinformatics, scientists are faced with an interesting conundrum. We now have access to sequences for countless numbers of potential genes without ever testing their functions in the cell. The Basic Local Alignment Search Tool (BLAST) has become such an integral tool for analysis, we as scientists often forget that very few experiments have actually been done to elucidate and characterize these genes. The original papers (Altschul et al., 1997, Altschul et al., 1990) describing BLAST continue to be some of the most cited scientific literature articles (Geer, 2006). Many of the algorithms used with the original BLAST have been implemented on several genome websites to enhance the search for potential genes of interest (Geer, 2006).

Although this vast sea of information is available with very little characterization, it does provide a multitude of benefits that are enhancing research. For example, BLAST alignment gives greater identification of possible relationships between different species and genes, allowing for greater efficiency in the design of experiments. Also, the cost effectiveness of the BLAST program allows science to move at lightning speed (Geer, 2006). One cannot truly grasp the efficiency of how this program has enhanced research until we look at the breadth of just one research topic such as ergosterol biosynthesis. In 1978, the first sterol mutants in C. reinhardtii were isolated by selecting for single colonies resistant to nystatin (Bard et al., 1978, Salimova et al., 1999). In 1981, a
chromatographic analysis was done to identify the major sterols and fatty acids in C. reinhardtii (Gealt et al., 1981), in 1982, sterol synthesis was studied during the cell cycle by Janero et al., and finally in 1999, Salimova et al., identified three sterol mutants that were deficient in C24(28)reductase (Salimova et al., 1999, Janero et al., 1982). This paper also identified a possible pathway for the synthesis of ergosterol and 7-dehydroporiferasterol based on enzyme function as seen in Figure 3.1 (Bard et al., 1978, Salimova et al., 1999). However, this pathway does not show potential substrates and precursors for earlier biosynthetic steps of the ergosterol pathway (Bard et al., 1978, Salimova et al., 1999). Unfortunately, from that point on very little research has been published in this field. During these earlier years, studying specific genes of the pathway was difficult because the BLAST tool was not yet available with the present day conveniences of reduced cost, algorithmic efficacy, and time efficiency, and as well as the Chlamydomonas genome was not yet sequenced. With the Chlamydomonas genome now 95% sequenced (Merchant et al., 2007), the ergosterol biosynthetic pathway can be predicted, and experiments designed to characterize the functions of these genes. Using BLAST alignment tools and bioinformatics technology, determinations of possible genes involved in ergosterol biosynthesis in C. reinhardtii can be made. Elucidation of the ergosterol pathway in C. reinhardtii will provide more insight into the significance of this lipid molecule and provide the groundwork for possible manipulation of these enzymes for further analysis and study.

3.2 Results

C. reinhardtii has been described as having two major sterols in its membrane, ergosterol and 7-dehydroporiferasterol (Gealt et al., 1981). By making use of BLAST sequence alignment tools on the NCBI database, several enzymes in the yeast pathway
Figure 3.1 The Ergosterol Pathway in *C. reinhardtii*. This diagram shows the reactants, enzymes and precursors to ergosterol and 7-dehydroporiferasterol in *C. reinhardtii* as described in (Bard *et al.*, 1978, Salimova *et al.*, 1999). Gene names have been added to the original pathway as well as the gene responsible for the alkylation of the second branch of the pathway that produces 7-dehydroporiferasterol.
for ergosterol biosynthesis were analyzed for sequence similarity in *C. reinhardtii*. Information about the structure and function of these enzymes was used to identify possible genes of interest involved in ergosterol biosynthesis in *C. reinhardtii*.

Sequences of ergosterol biosynthetic enzymes were gathered from the *Saccharomyces* Genome Database (www.yeastgenome.org). These sequences were then aligned against the JGI *Chlamydomonas* Genome Version 4.0 database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) using the BLAST algorithm to identify sequences with similarity and identity. For each enzyme two or three genes showed significant homology. Only those sequences with the best E-value were chosen as potential candidates for each of the genes (Table 3.1). An E-value is a statistical number denoting a high sequence similarity between a query and subject sequence. Some of the genes of the ergosterol biosynthetic pathway were then named and annotated by Dr. J.V. Moroney as part of his contribution to the *Chlamydomonas* JGI Annotation Team (Merchant et al., 2007). In addition, we also determined the exon number, length of genomic and cDNA, as well as putative protein function (Table 3.2) (Merchant et al., 2007), so as to better design a proposed pathway for ergosterol biosynthesis in *C. reinhardtii* (Figure 3.2). Each gene is described based on previous research discoveries in *S. cerevisiae*. Hopefully, this information will give scientists some groundwork to design more efficient experiments to begin characterization of these genes.

3.2.1 ERG20

Using the *S. cerevisiae* amino acid sequence to search the *Chlamydomonas* genome database, one gene was identified with an E-value of e-102 (Table 3.1). Located on Chromosome 3, ERG20 was identified as a farnesyl diphosphate synthetase (Table 3.2) based on its function yeast. The proposed reaction catalyzed by Erg20p is explained
Table 3.1. *Chlamydomonas* Ergosterol Genes and Genome Location.

<table>
<thead>
<tr>
<th>Chlamydomonas Ergosterol Gene</th>
<th>E-value</th>
<th>Genome Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG20 (FPPS)</td>
<td>e-102</td>
<td>Chromosome 3: 7017328-7021524</td>
</tr>
<tr>
<td>ERG9</td>
<td>2e-75</td>
<td>Chromosome 3: 3398442-3402247</td>
</tr>
<tr>
<td>ERG1</td>
<td>5e-69</td>
<td>Chromosome 15: 2524204-2531216</td>
</tr>
<tr>
<td>ERG7 (CAS1)</td>
<td>e-103</td>
<td>Chromosome 1: 1873239-1882768</td>
</tr>
<tr>
<td>ERG6 (SMT1)</td>
<td>9e-63</td>
<td>Chromosome 12: 2020653-2024227</td>
</tr>
<tr>
<td>CP1*</td>
<td>2e-86</td>
<td>Chromosome 16: 1259701-1263345</td>
</tr>
<tr>
<td>ERG11 (CYP51G1)</td>
<td>5e-59</td>
<td>Chromosome 2: 2409617-2414200</td>
</tr>
<tr>
<td>CDI1*</td>
<td>6e-38</td>
<td>Chromosome 12: 8843417-8845452</td>
</tr>
<tr>
<td>ERG25</td>
<td>7e-13</td>
<td>Chromosome 6: 1665867-1669592</td>
</tr>
<tr>
<td>ERG26</td>
<td>3e-39</td>
<td>Chromosome 10: 3130700-3133000</td>
</tr>
<tr>
<td>ERG28</td>
<td>4e-08</td>
<td>Chromosome 13: 878655-881039</td>
</tr>
<tr>
<td>ERG3</td>
<td>8e-25</td>
<td>Chromosome 16: 2115065-2117963</td>
</tr>
<tr>
<td>ERG5 (CYP710B1)</td>
<td>2e-52</td>
<td>Chromosome 18: 1235761-1240121</td>
</tr>
<tr>
<td>ERG4/24</td>
<td>3e-36</td>
<td>Chromosome 2: 482034-486648</td>
</tr>
</tbody>
</table>

*Derived from pBLAST alignment of *CP1* and *HYDRA1* from *Arabidopsis thaliana*. Higher plant nomenclature denoted in parentheses.

Table 3.2. *Chlamydomonas* Ergosterol Gene Information and Protein Function.

<table>
<thead>
<tr>
<th>Chlamydomonas Ergosterol Gene</th>
<th>Number of Exons</th>
<th>Approximate Size of Genomic DNA</th>
<th>Approximate Size of cDNA</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG20 (FPPS)</td>
<td>12</td>
<td>1.7 kb</td>
<td>1.1 kb</td>
<td>farnesyl disphosphate synthetase</td>
</tr>
<tr>
<td>ERG9</td>
<td>10</td>
<td>1.1 kb</td>
<td>1.1 kb</td>
<td>farnesyl-disphosphate farnesyl transferase</td>
</tr>
<tr>
<td>ERG1</td>
<td>12</td>
<td>2.1 kb</td>
<td>1.6 kb</td>
<td>monooxygenase/ hydroxylase</td>
</tr>
<tr>
<td>ERG7 (CAS1)</td>
<td>20</td>
<td>2.3 kb</td>
<td>2.3 kb</td>
<td>cycloartenol synthase</td>
</tr>
<tr>
<td>ERG6 (SMT1)</td>
<td>6</td>
<td>1.8 kb</td>
<td>1.2 kb</td>
<td>sterol C-24 methyltransferase</td>
</tr>
<tr>
<td>CP1*</td>
<td>8</td>
<td>1.9 kb</td>
<td>0.8 kb</td>
<td>cycloartenol cycloisomerase</td>
</tr>
<tr>
<td>ERG11 (CYP51G1)</td>
<td>10</td>
<td>2.7 kb</td>
<td>1.5 kb</td>
<td>sterol 14- desaturase</td>
</tr>
<tr>
<td>CDI1*</td>
<td>5</td>
<td>1.4 kb</td>
<td>0.6 kb</td>
<td>C(8,7) sterol isomerase</td>
</tr>
<tr>
<td>ERG25</td>
<td>6</td>
<td>1.3 kb</td>
<td>0.9 kb</td>
<td>sterol desaturase</td>
</tr>
<tr>
<td>ERG26</td>
<td>1</td>
<td>2.3 kb</td>
<td>1.2 kb</td>
<td>C-3 sterol dehydrogenase</td>
</tr>
<tr>
<td>ERG28</td>
<td>5</td>
<td>1.5 kb</td>
<td>0.4 kb</td>
<td>not annotated</td>
</tr>
<tr>
<td>ERG3</td>
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<td>1.9 kb</td>
<td>1.1 kb</td>
<td>C-5 sterol desaturase</td>
</tr>
<tr>
<td>ERG5 (CYP710B1)</td>
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<td>2.2 kb</td>
<td>1.5 kb</td>
<td>C-22 sterol desaturase</td>
</tr>
<tr>
<td>ERG4/24</td>
<td>7</td>
<td>2.3 kb</td>
<td>1.3 kb</td>
<td>C- 24(28) sterol reductase</td>
</tr>
</tbody>
</table>

*Derived from pBLAST alignment of *CP1* and *HYDRA1* from *Arabidopsis thaliana*. Higher plant nomenclature denoted in parentheses.
Figure 3.2. Proposed Pathway for Ergosterol Biosynthesis in *C. reinhardtii*. Sterol pathway showing the reactants and enzymes that catalyze the steps to synthesize ergosterol in *C. reinhardtii*. Based on EST data from JGI VERSION 4.0, *Chlamydomonas* lacks similar sequences for *ERG27* and *ERG2* from *S. cerevisiae*, but it does show sequence homology with *CPI1* and *CDI1*, genes involved in sterol biosynthesis in *Arabidopsis thaliana*. This working model may change as these genes are characterized.
in Figure 3.3. The length of the genomic DNA and cDNA are approximately 1.7 kb and 1.1 kb, respectively. \textit{ERG20} has 12 exons (Figure 3.4B) and a coding sequence of 1083 nucleotides (Figure 3.4A) which encodes a 360 amino acid polypeptide (Figure 3.4C). \textit{ERG20} encoded a protein that also showed homology with the farnesyl diphosphate synthetase of \textit{A. thaliana} (Accession No. CAA53433), as well (Figure 3.5). \textit{ERG20} has been characterized by functional complementation in yeast (Delourme \textit{et al.}, 1994). \textit{C. reinhardtii} Erg20p has 52% sequence identity to farnesyl diphosphate synthetase from \textit{A. thaliana} and 49% identity to Erg20p in \textit{S. cerevisiae}. The \textit{Arabidopsis} gene for this important enzyme in isoprenoid and sterol biosynthesis, \textit{ERG20}, is found on Chromosome 3 in \textit{C. reinhardtii} and has 12 exons. The gene model is completely supported by EST data. The predicted 360 amino acid protein is soluble and lacks transmembrane helices.

In yeast, the \textit{ERG20} homolog, is described as an essential gene that feeds C10 precursors to several lipid pathways including sterol biosynthesis (Anderson \textit{et al.}, 1989). The enzyme functions to couple IPP to dimethylallyl diphosphate and geranylphosphate to produce C15 metabolites that can enter the sterol biosynthetic pathway (Anderson \textit{et al.}, 1989). Two yeast \textit{ERG20} mutants were identified and as a result produce geraniol and farnesol which are toxic alcohols (Chambon \textit{et al.}, 1990).

\textbf{3.2.2 ERG9}

Erg9p in \textit{C. reinhardtii} is described as a farnesyl-diphosphate farnesyl transferase. The primary function of the protein is to join two farnesyl pyrophosphates to form squalene (Figure 3.3). This biosynthetic branch point is thought to regulate the flow of isoprene units into the sterol pathway (Jennings \textit{et al.}, 1991). Erg9p in yeast contains a
Figure 3.3. Schematic diagram of the elongation of squalene. Isoprene units are joined together by Erg20p and Erg9p to form squalene.
Figure 3.4. Full Length cDNA Sequence and Gene Structure of ERG20 and Amino Acid Sequence of Erg20p. A: Full length cDNA sequence of the *C. reinhardtii* ERG20 gene. Start and Stop codons are shown in the green boxes, respectively. B: Gene structure of the *C. reinhardtii* ERG20 gene. Start and Stop codons are denoted by the black vertical bars. C: Amino acid sequence of the Erg20 protein.
Figure 3.5. Multiple Alignment of ERG20. Multiple sequence alignment of *C. reinhardtii* Erg20 protein with homologs from *A. thaliana* and *S. cerevisiae*. The “*” denotes identical amino acid residues. The “:” denotes conserved residues. The “.” denotes residues that are semi-conserved.
PEST consensus motif that is thought to be a potential site for phosphorylation of serine/threonine residues for the purpose of ubiquitination (Rogers et al., 1986). Erg9p lacks the PEST consensus motif in *C. rienhardtii*, but it does show sequence similarity with another portion of the yeast Erg9 protein. This particular sequence, MMGLFLQKTNIRDYNEDLVDRGR, is a putative prenyl substrate motif (Jennings et al., 1991) (Figure 3.6). *ERG9* is predicted to encode a soluble 378 amino acid protein and the gene structure, located on Chromosome 3, and has 10 exons and 9 introns. While, the genome produces a strong match for this gene with an E-value of 2e-75, the EST data in support of this gene model are very weak.

MGKLGELLSHPNEIMPLRLMYRAAKRATKLPKDPLAFCGMYGMLNDVRSFAIVIQQLPNPLRDSSICVFYL
VLRLGDTVDMDALDVDFKLPERRTHEFYDRTMECGYNHYKRLMAQFGVVTVNLPAALPPPHQAVIA
DITRMGMYGMAEFIEKEVTVADYDKYCHYVAGLVGIGLSQLFAASGLEANSSGTEEDLANHMGLFLQKT
NIIRDYLEDILPAPRMFWPRDIWGKYAPALADFKEPANRGAAVRCCLNDMITALRHPYCLAYMEQLR
HVMVFRCAPQIMAAGTLAACYNNGRVFEGVKMRRGETARIFETCNDLGDLITYTWFLQFLAAMEAKVD
TPDSLPLTRQRIAEARKCACAALKTEVSV

**Figure 3.6. Full Amino Acid Sequence of Erg9p.** The amino acid sequence of Erg9 protein. The putative prenyl substrate motif is highlighted in the red box.

### 3.2.3 ERG1

*ERG1* is highly conserved among eukaryotic organisms producing sterols. In *Saccharomyces cerevisiae*, Erg1p is classified as a squalene epoxidase and is responsible for catalyzing a major reaction in the ergosterol biosynthetic pathway (Jandrositz et al., 1991). The essential role of Erg1p is to facilitate the reaction responsible for introducing a molecular oxygen at the 2,3 position of squalene to produce squalene epoxide (Ryder, 1990) (Figure 3.7). By eliminating *ERG1* in yeast, auxotrophic mutants are created.
Without an ergosterol supplement in the media, under anaerobic conditions these mutations are lethal (M'Baya et al., 1989). ERG1 is also a target for the antifungal class of drugs called terbinafines (Ryder, 1991, M'Baya et al., 1989).

Like yeast and Arabidopsis, Chlamydomonas reinhardtii possesses a similar protein called Erg1p. It is also characterized in the JGI Chlamy Version 4.0 Database as having oxidoreductase activity. The protein is predicted to have two transmembrane helices. Protein family alignment (Pfam) of the protein sequence for C. reinhardtii reveals a domain structure with similarities to a FAD dependent oxidoreductase. Found on Chromosome 15, ERG1 has 12 exons. Its genomic DNA is about 2.1 kilobases while its cDNA is 1.6 kilobases. The gene model for ERG1 has partial EST support, there is a portion of the gene where there is no EST data. It must be noted that the ERG1 gene is not annotated in the JGI Chlamy Version 4.0 Database.

3.2.4 ERG7- Cycloartenol Synthase

Cycloartenol synthase catalyzes the reaction from 2,3 epoxysqualene to cycloartenol. This critical step is a major evolutionary branch point between photosynthetic eukaryotes and nonphotosynthetic organisms like yeast (Benveniste, 2004) (Figure 3.7). For example, in S. cerevisiae 2,3-epoxysqualene is cyclized to lanosterol (Benveniste, 1986) by a 2,3-OS lanosterol cyclase or Erg7p. Functional complementation of yeast mutants lacking lanosterol synthase with A. thaliana cycloartenol syntheses led to the discovery of CAS1 (Corey et al., 1993). Very interestingly, A. thaliana cycloartenol synthase has been shown to produce lanosterol when point mutations are present (Y410T or H477N) (Benveniste, 2004, Segura et al., 2002, Herrera et al., 1998, Hart et al., 1999).

Lanosterol synthase and cycloartenol synthase are quite similar with more than
Figure 3.7. Schematic diagram showing the biochemical reactions from squalene to cycloartenol and lanosterol. ERG1 catalyzes the conversion of squalene to squalene epoxide. This step is highly conserved among eukaryotic organisms. The branch between nonphotosynthetic eukaryotes and photosynthetic organisms shows the differentiation in structure among higher plants, algae, fungi and mammals. ERG7 and CAS1 catalyze these reactions.
35% identity between the genes. Summons et al. has identified key amino acid differences between lanosterol synthase and cycloartenol synthase (Summons et al., 2006). Amino acids at position T/Y381, C/Q/H449, and V/I453 are conserved among lanosterol and cycloartenol synthase, while amino acid D455 is thought to be a highly conserved catalytic residue (Summons et al., 2006). Looking at these specific amino acids, C. reinhardtii sequence aligns well with the higher plant sequence for cycloartenol synthase (Figure 3.8). The identification of CAS1 supports the idea that C. reinhardtii does follow a sterol biosynthetic pathway very similar to that of higher plants. The gene is found on Chromosome 1. It has 20 exons (Figure 3.9) and sequence alignment reveals a 34% similarity between lanosterol synthase, Erg7p, in yeast and 58% sequence similarity with cycloartenol synthase in A. thaliana (Figure 3.10). While CAS1 has been annotated, there is very little EST data to support this model.

3.2.5 ERG6- Sterol-C- Methyltransferase

Ergosterol biosynthesis is thought to be highly conserved across several different species, including humans, but the biosynthetic step that ERG6 catalyzes is not found in Homo sapiens (Jensen-Pergakes et al., 1998). Erg6p in yeast is a Δ(24)-sterol C-methyltransferase (Gaber et al., 1989). It is thought to catalyze the conversion of zymosterol to fecosterol by methylating position C-24 in yeast. In higher plants, like A. thaliana, ERG6 is called SMT1 and catalyzes two steps. It is responsible for both the conversion of cycloartenol to 24- methylene cycloartenol (Wojciechoswki et al., 1973), as well as catalysis of 24-methylenophenol to 24- ethylenophenol (Fonteneau et al., 1977). ERG6 is responsible for these two separate steps in sterol biosynthesis in higher plants as well as C. reinhardtii. The primary difference between vertebrate sterols and those of fungi and higher plants is an extra alkyl group on carbon 24 (Nes, 2000).
Figure 3.8. Multiple Alignment of Lanosterol and CycloartenolSynthase Across Species. The colored amino acids are denoted as being diagnostic between LAS and CAS. At position 381 threonine is conserved in LAS but tyrosine is conserved in CAS. Likewise in position 449 is a C or Q in LAS and an H in CAS. Position 453 is a V in CAS and an I in LAS. While position 455 is conserved among both as the catalytic amino acid. The crystal structure of the oxidosqualene cyclase in *H. sapiens* has been published (Thoma *et al.*, 2004).

Figure 3.9. Exon- Intron Structure of CAS1 in *C. reinhardtii*.
| CAS1 | ----MKFISAGTGGGLLRSMLGNKGRQTYWEVPAGPTQERAEAERLEEFECTDNKDH 56 |
| CAS | ----MKADLQAGTGGGLLRSMLGNKGRQTYWEVPAGPTQERAEAERLEEFECTDNKDH 56 |
| CAS | ----MKADLQAGTGGGLLRSMLGNKGRQTYWEVPAGPTQERAEAERLEEFECTDNKDH 56 |
| ERG7 | MTEFYSDTGILTPDRLPRWLRDLERGSESWEYLTPQQAND-PSTPRAWLLQDPKFPQ 59 |
| CAS | NGSDELLRLQADRRRAKKSPPSGPVDFAPAD----REEERLHGAISFYELQQED-GH 113 |
| CAS | NGSDELLRLQADRRRAKKSPPSGPVDFAPAD----REEERLHGAISFYELQQED-GH 113 |
| CAS | NGSDELLRLQADRRRAKKSPPSGPVDFAPAD----REEERLHGAISFYELQQED-GH 113 |
| ERG7 | PHP---------------ERKNHSDPSFAPDACHN------------------GASFKLQPEQDOSI 94 |
| CAS1 | FPQDYGPGFLLLPGPVITLYCAGDQIPFSGAVKEAKLVGNHNQ-DEGGFGHLHIEGS 172 |
| CAS | FPQDYGPGFLLLPGPVITLYCAGDQIPFSGAVKEAKLVGNHNQ-DEGGFGHLHIEGS 172 |
| CAS | FPQDYGPGFLLLPGPVITLYCAGDQIPFSGAVKEAKLVGNHNQ-DEGGFGHLHIEGS 172 |
| ERG7 | TFMGFLVNYVMMALLGLAASEEP----LVCAVREWMHARGTITSWKFWAVLVGYSQGD 230 |
| CAS1 | ERG7 | TMFGSVLNYVTLRLLGDENMDIEEKSALHIEGGS 172 |
| CAS | ERG7 | TMFGSVLNYVTLRLLGDENMDIEEKSALHIEGGS 172 |
| CAS | ERG7 | TMFGLVNYVIMRLGPRPLSPQKPGFSPQLFQ 302 |
| ERG7 | TVFGTVLNYVIMRLGPRPLSPQKPGFSPQLFQ 302 |
| CAS1 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | VNSAPPETQWLLYSQPGKLAVLHFQGKPQ 380 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS1 | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| CAS | ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |
| ERG7 | MNFLPFLWLLHLHNFQGTGMLHPVFCRVMVLPSYVVGKRCGTCQGETFLTAIRGE 290 |

**Figure 3.10. Multiple Alignment of Erg7p and Cas1p.** Multiple sequence alignment of _C. reinhardtii_ cycloartenol synthase protein with homologs from _A. thaliana_ and _S. cerevisiae_. The “*” denotes identical amino acid residues. The “:” denotes conserved residues. The “.” denotes residues that are semi-conserved.
Erg6p or sterol-C-methyltransferase is responsible for the catalysis of this side chain.

Preliminary information about the gene in *C. reinhardtii* reveals that *SMT1* is located on Chromosome 12. In *C. reinhardtii*, Smt1p is a soluble protein and is a member of the SAM dependent methyltransferase protein family ([http://pfam.sanger.ac.uk](http://pfam.sanger.ac.uk)). SAM dependent methyltransferases are responsible for the transfer of methyl groups to other biological molecules for sterol biosynthesis and even DNA repair ([http://pfam.sanger.ac.uk](http://pfam.sanger.ac.uk)). *SMT1* in *C. reinhardtii* has a cDNA sequence of 1.2 kilobases, and its intron-exon structure spans the genome at 1.8 kilobases. Bioinformatics evidence reveals only one copy of *SMT1* in *Chlamydomonas* in contrast to the three found in Arabidopsis. There is strong EST support for this gene. The EST data also indicated that two forms of the Smt1 protein may exist in *Chlamydomonas* as there appears to be alternative splicing with one form of the gene.

### 3.2.6 ERG25, ERG26, ERG28 – Sterol C-4 Demethylation Complex

The C-4 demethylation complex includes proteins Erg25p, Erg26p, and Erg27p in yeast (Mo *et al.*, 2004). The end product sterols are converted from cycloartenol by way of the removal of two methyl groups at position 4 and 14 (Benveniste, 2004a). It has been established that three enzymes are used to convert 24-methylene cycloartenol to cycloeucalenol in maize embryos (Pascal *et al.*, 1993). The first enzyme Erg25p serves as a C-4 methyl oxidase (Bard *et al.*, 1996). Erg26p is a C4-decarboxylase/C3-dehydrogenase, and finally Erg27p completes the demethylation by its 3-keto reductase activity (Pascal *et al.*, 1994). These enzymes also catalyze the conversion of 24-methylene or 24-ethylidene lophenol to episterol and avenosterol respectively in *A. thaliana* (Benveniste, 2004a).

It must be noted however that *A. thaliana* does not show any homologous protein
matches for Erg27p. Blastp search of the JGI genome database also reveals that *C. reinhardtii* also lacks an *ERG27* gene. *ERG25* in *Chlamydomonas* is found on Chromosome 6 and has 6 exons. It is predicted to have 4 transmembrane domains.

*ERG26* is identified as a 2.3kb gene on Chromosome 12. While these genes have not been characterized in *C. reinhardtii*, Smop (sterol-4α-methyl oxidase or Erg25p) from *Arabidopsis* was identified by functional complementation in yeast *ERG25* mutants (Darnet *et al.*, 2001).

From an evolutionary perspective, it is still largely unknown how or why this methylation is possible without the 3-keto reductase activity of Erg27p. In 2003, Mo *et al*. showed that *ERG27* was essential and required for *ERG7* activity in yeast (Mo *et al*., 2003). Although Erg27p has not been identified in *A. thaliana* or *C. reinhardtii*, *SMO* genes offer new information about other possible enzymes that may be performing keto-reductase activity in higher plants. When *SMO1* and *SMO2* gene expression is reduced in *Nicotiana benthamina*, 24-methylene cycloartenol and 24-methylene and 24-ethylidene lophenol accumulate as precursors (Schaller, 2004). However, there is an additional protein identified in yeast that is thought to stabilize and anchor the sterol C-4 demethylation enzyme complex to the ER (Mo *et al*., 2004) (Figure 3.11). This protein is called Erg28p and is conserved in *A. thaliana* as well as *C. reinhardtii* (Gachotte *et al*., 2001). Erg28p is found on Chromosome 13 and has 5 exons. While this gene is not annotated and is poorly characterized in the JGI Genome Version 4.0, an NCBI blast of non-redundant protein sequences does reveal a match to the *ERG28* superfamily.

In the current model (Mo *et al*., 2004), Erg28p is thought to bridge between the demethylation complex (Erg25p, Erg26p, Erg27p) and the other sterol biosynthetic proteins such as Erg3p and Erg6p (Figure 3.11A). Another enzyme found in close
proximity to the Erg28p bridge is Erg2p. There were no sequences identified by a blastp search of yeast ERG2 to *Chlamydomonas* (Figure 3.11B). Comparision of the yeast pathway to that of the *Arabidopsis* pathway reveals that this gene may not be necessary in order to achieve the demethylation steps and the production of the final end steps of ergosterol biosynthesis in *C. reinhardtii* (Figure 3.11C). There was sufficient EST support for *ERG25*, *ERG26* and *ERG28*. However, because *ERG27* was not confirmed after the BLASTp search, there was no EST support for this gene.

### 3.2.7 CPI1- Cyclopropyl Sterol Isomerase

*CPI1* was a very important gene to identify by bioinformatics analysis. This gene is found primarily in the plant kingdom and denotes use of the cycloartenol pathway versus the lanosterol pathway. Cyclopropyl sterol isomerase functions to open a cyclopropane ring in culcoeucalenol to catalyze the production of obtusifoliol (Rahier *et al.*, 1989, Heintz & Benveniste, 1974) (Figure 3.12). This gene is primarily restricted to the plant kingdom. Many studies have looked at agricultural pesticide targeting to *CPI1* in higher plants (Benveniste, 1986), but this gene has not been analyzed in *C. reinhardtii*. With the exception of the bioinformatics data presented here, scientists have yet to investigate the role of this gene in the model system. It has been found that *CPI1* is a drug target for two parasitic amoebae, *Acanthamoeba* and *Naegleria* (Raederstorff & Rohmer, 1985, Raederstorff & Rohmer, 1987). Humans and other vertebrate organisms do not have this gene and therefore are unlikely to be affected by drugs specifically targeting *CPI1*.

*CPI1* is located on Chromosome 16. The genomic DNA spans 1.9 kb and the cDNA is 0.8 kb in size (Figure 3.13A). *CPI1* gene has 8 exons, (Figure 3.13B), and the protein is 276 amino acids long (Figure 3.13C). While very little is known about the
Figure 3.11. Schematic diagram of the Sterol C-4 Demethylation Complex. (A) Erg28p anchors the complex in yeast and forms a bridge between Erg27p and Erg6p in this protein-protein interaction. Modified from (Mo et al., 2004). (B) Erg27p and Erg2p are absent in the genomes of both *Chlamydomonas* and *Arabidopsis*. (C) A proposed schematic diagram of Erg28p bridging Erg26p to Erg6p.
gene in *C. reinhardtii*, the presence of strong EST support for *CPII* does give sufficient data and support for the idea of a more plant-like pathway from squalene to episterol.

### 3.2.8 ERG11- Lanosterol 14α-Demethylase /CYP51G1- Obtusifoliol 14α-Demethylase

*ERG11* and *CYP51G1* are responsible for two different conversions in higher plants and fungi. Lanosterol is the substrate of Erg11p in yeast; in contrast, obtusifoliol is the substrate in higher plants (Delye *et al.*, 1998, Taton & Rahier, 1991). Even though these two enzymes utilize different substrates, they both have the conserved protein sequence FGxGRHxCIG that represents a Cytochrome p450 cysteine-heme iron ligand binding signature (Figure 3.14). While Cytochrome p450 proteins are highly substrate specific, heterologous complementation from yeast to higher plant is often very difficult. Even though a higher plant to yeast *ERG11* complementation has been reported, the authors had to optimize the REDOX environment by overexpressing an additional cytochrome p450 protein in order to assist in the direct complementation of the higher plant *ERG11* in yeast (Cabello-Hurtado *et al.*, 1999).

BLASTP alignment with the yeast Erg11 protein sequence to the *Chlamydomonas* genome revealed that *CYP51G1* is found on Chromosome 2 and is predicted to encode a sterol 14-desaturase. With 10 exons, the gene spans 2.7 kb and its cDNA is 1.5 kb long. The EST support for *CYP51G1* is very strong. The sequence of Cyp51G1p is 52% similar to the sequence of Cyp51G1 for Arabidopsis, while, it is only 28% similar to Erg11p in yeast. But, all three sequences have the Cytochrome p450 cysteine-heme iron ligand binding signature (Figure 3.14).

### 3.2.9 ERG4/24- C-24(28) Reductase/C-14 Sterol Reductase

*ERG4* and *ERG24* are two separate genes in *S. cerevisiae*. Erg4 protein in *C.
Figure 3.12. Schematic diagram of the chemical conversion of cycloeucalenol to obtusifoliol. CPII helps open the cyclopropane ring of cycloeucalenol to form obtusifoliol.
Figure 3.13. **Full Length cDNA Sequence and Gene Structure of CPI1 and Amino Acid Sequence of Cpi1p.**

A: Full length cDNA sequence of the *C. reinhardtii* CPI1 gene. Start and Stop codons are shown in the green boxes, respectively.

B: Gene structure of the *C. reinhardtii* CPI1 gene. Start and Stop codons are denoted by the black vertical bars.

C: Amino acid sequence of the Cpi1 protein.
reinhardtii is 26% similar to Erg24p in yeast, and Erg4p is found on Chromosome 7 while ERG24 is found on Chromosome 14. Each have their own function, but BLASTP search of the Chlamydomonas genome reveals only one gene for both ERG4 and ERG24. In C. reinhardtii, ERG4/24 is located on Chromosome 2. The protein is predicted to have 8 transmembrane helices, while, the gene has 7 exons and 6 introns. C. reinhardtii Erg4/24p is 30% similar to yeast Erg4p and 37% similar to yeast Erg24p (Figure 3.15). The dual functions of Erg4/24p are sterol C-24(28) reductase and C-14 sterol reductase activity. The EST support is very strong for this gene. ERG4/24 in C. reinhardtii belongs to the ergosterol biosynthesis ERG4/24 family according to Pfam predictions and NCBI Blast predictions. The major function of this protein is to reduce double bonds in the precursors of ergosterol (Lai et al., 1994).

While BLASTp searches with Erg4p and Erg24p from S. cerevisiae reveals only one gene corresponding to both in C. reinhardtii, A. thaliana does possess a C-14 sterol reductase. The gene known as FACKEL (At3g52940) encodes a C-14 sterol reductase that catalyzes the C-14 demethylation of obtusifoliol (Benveniste, 2004b). Fackelp has been characterized, and the fk mutation in A. thaliana promotes the accumulation of $\Delta^{8,14}$ sterol intermediates (Klahre et al., 1998). A. thaliana also has a $\Delta^{24,28}$ sterol reductase, Dwf1p. DWF1 performs several steps in the A. thaliana sterol pathway, but its primary role is the isomerization and reduction of bonds located at C-24,28 (Klahre et al., 1998). Unfortunately, at this time there is no purified protein of DWF1.

### 3.2.10 CDI1- $\Delta^8$-$\Delta^7$ Sterol Isomerase

During sterol biosynthesis, the methyl group of Carbon 14 is removed in higher plants, algae and fungal species. Following this step, $\Delta^7$ sterols are produced by a $\Delta^8$-$\Delta^7$ isomerase (Benveniste, 2004). Erg2p is the equivalent $\Delta^8$-$\Delta^7$ isomerase, however, upon a
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
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<td>Chlamydomonas</td>
<td>-----MLPPELAVLDKVLSLSPVVLVALGSAVLILAVGRLFVINVFNLPSKR-----PPW 52</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>-----MLDSE-----NKLKTLTGLIVATLV1AKLIFSFTDSKKKRLF-------PTLK 44</td>
</tr>
<tr>
<td>Yeast</td>
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</tr>
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Figure 3.14. Sequence similarity between ERG11 protein sequences in Chlamydomonas reinhardtii, Arabidopsis thaliana, and Saccharomyces cerevisiae. Identical protein sequences are denoted by an asterisk. The Erg11p sequences show a conserved protein sequence FGxGRHxCIG that is highlighted in blue. This motif represents a Cytochrome p450 cysteine-heme iron ligand binding signature. The “*” denotes identical amino acid residues. The “.” denotes conserved residues. The “.” denotes residues that are semi-conserved.
Blastp search of the *Chlamydomonas* genome, no sequences homologous to *ERG2* are found. When taking the same *ERG2* sequence and doing a Blastp search of the *Arabidopsis* genome, no equivalent matches are found. However, another gene *HYDRA1* in *A. thaliana* has been identified to have a similar function to *ERG2* in yeast despite its low sequence similarity. *HYDRA1* gene does complement the genetic phenotype of erg2Δ mutants in *S. cerevisiae* (Benveniste, 2004a, Benveniste, 2004b). Blastp search of the *Chlamydomonas* genome with Hydra1p does reveal one gene annotated as *CDI1*. This gene has 5 exons and is located on Chromosome 12. Although these genes are annotated as functioning as Δ8-Δ7 isomerases, it must be noted that there is no experimental evidence in *C. reinhardtii* to support this claim. However, the EST support for this gene is extremely strong. When analyzing the sequence of Erg2p in yeast, Hydra1p in *Arabidopsis*, and Cdi1p in *Chlamydomonas*, there is very little sequence identity among the three sequences (Figure 3.16). There is a 37% similarity between Hydra1p and Cdi1p, while there is a 4% similarity between Cdi1p and Erg2p.

### 3.2.11 ERG3-C-5 Sterol Desaturase

*ERG3* has been well characterized in yeast as a C-5 sterol desaturase (Osumi *et al.*, 1979), as it catalyzes the Δ5- bond in the B-ring of ergosterol. This enzyme is thought to be sensitive to cyanide and needs iron as well as molecular oxygen for its function (Skaggs *et al.*, 1996). *ERG3* in yeast is also associated with NAD(P)H-cytochrome b/ cytochrome b5 (Osumi *et al.*, 1979). Deletion of *ERG3* in *Saccharomyces cerevisiae* produces haploid cells that overaccumulate the ergosterol precursor episterol.

*ERG3* in *C.reinhardtii* is located on Chromosome 16 of the genome and functions as a C-5 sterol desaturase. The genome database predicts that this particular protein has 3
Figure 3.15. Sequence similarity between *S. cerevisiae* Erg4p and Erg24p and *C. reinhardtii* Erg4/24p. The “*” denotes identical amino acid residues. The “:” denotes conserved residues. The “.” denotes residues that are semi-conserved.
Figure 3.16. Sequence similarity between Δ8-Δ7 isomerases in *Chlamydomonas reinhardtii*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. The “*” denotes identical amino acid residues. The “:” denotes conserved residues. The “.” denotes residues that are semi-conserved.
transmembrane helices. The first helix is thought to be located at amino acids 10-32, the second and third helix correspond to amino acids 110-132 and 191-213. According to Pfam analysis, Erg3 protein from *C. reinhardtii* belongs to the fatty acid hydroxylase superfamily. This superfamily of proteins includes C-5 sterol desaturases as well as C-4 sterol methyl oxidases. *ERG3* has 6 exons and 5 introns, and its protein sequence has similar sequence identity with Erg3p of *S. cerevisiae*. The EST support for this gene is very strong. This gene will be discussed further in Chapter 4.

### 3.2.12 *ERG5*

*ERG5* is considered to be a member of the cytochrome P-450 superfamily in yeast (Kelly *et al*., 1997). The primary function of this protein is the conversion of ergosta-5,7-dienol to ergosta-5,7,22,24-tetraen-3β-ol. As a C-22 sterol desaturase, Erg5p catalyzes insertion of a double bond in the sterol side chain and is thought to be susceptible to azole antifungal drugs (Aoyama *et al*., 1984). Bard *et al.* identified *C. reinhardtii* mutants lacking ergosterol (Bard *et al*., 1978). These mutations are consistent with a loss of *ERG5* activity. *ERG5* is found on Chromosome 18 and has 9 exons. Pfam predictions designate Erg5p has having a p450 conserved domain between amino acids 214 and 413. JGI reveals a molecular function of monoxgenase activity. There is partial EST support for this gene.

### 3.2.13 Ergosterol Biosynthetic Gene Expression

In 1978, the first sterol mutants were identified in *C. reinhardtii*. These mutants were described as being 22(23) desaturase mutants (Bard *et al*., 1978). *ERG5* is denoted as the C-22 desaturase gene in *C. reinhardtii* according to bioinformatics analysis. *ERG5* catalyzes two steps in the sterol pathway of *C. reinhardtii*. Erg5p helps to introduce double bonds in ergosta-5,7,24(28)-tri-enol and stigmasta-5,7,24(28)-tri-enol to produce
ergosta-5,7,22, 24(28)–tetratenol and stigmasta-5,7,22, 24(28)-tetratenol. Upon investigation of one of these original sterol mutants, quantitative real time PCR (Q-RT PCR) revealed that cc652 does lack expression of ERG5 (Figure 3.17). While, it is not known what part of the gene the point mutation exists, the Q-RT PCR results corroborate the original work that cc652 has a defective ERG5 gene (Bard et al., 1978).

In 1982, Janero et al., were the first to analyze cell cycle variations in sterol biosynthesis in Chlamydomonas. Their analysis revealed that sterol biosynthesis was highly active during the light period while there was a sharp decrease during the dark period. They also demonstrated that there is a marked increase of sterol synthesis during the transition from the dark to the light period by some 30 fold. With this information, it was decided that an analysis of specific ergosterol biosynthetic genes during membrane biogenesis of reflagellation was in order. Reflagellation is the process by which the flagella can regenerate after manual or chemical excision such as pH shock. Analysis of several key genes in sterol biosynthesis showed some sterol biosynthetic gene increases in expression during reflagellation (Figure 3.18).

It was of interest that ERG11 showed the greatest relative expression after 30 minutes of reflagellation. Erg11p is responsible for the conversion of cycloeucalenol to obtusifoliol in higher plants (Benveniste, 2004). The Erg11 enzyme is also extremely substrate specific. ERG3, ERG4, ERG5, ERG6, and ERG7 show moderate increases in relative expression during reflagellation compared to ERG11. These genes are responsible for some key steps in the pathway as described earlier, but it appears as though these genes turn on after 30 minutes of reflagellation, and then their expression returns to levels seen before the loss of the flagella. However, unlike ERG3, ERG4, ERG5 and ERG7, ERG6 stays active throughout the duration of the entire reflagellation.
Figure 3.17 Q-RT comparative analyses between wild type cells and a well-characterized sterol mutant cc652. In the experiment, the expression of each sterol gene wild type cells was set to 1 and the expression of the mutants was compared. The asterisk (*) denotes the absence of ERG5 expression.
cycle of 120 minutes. When looking at the pathway, it must be noted that ERG6 is responsible for catalyzing several steps in the ergosterol biosynthetic pathway. This gene may remain active to express more protein to catalyze these multiple steps in the pathway. Finally, ERG25 and ERG26 were also analyzed to identify their levels of expression during reflagellation, and it appears that these genes are constitutively expressed. It must be noted that these two genes are a part of the C-4 demethylation complex and interact in a multi-step process to remove methyl groups from carbon 4 of the sterol nucleus. These observations led me to investigate whether C. reinhardtii “Bald” mutants that completely lacked flagella showed any changes or absences of these ergosterol biosynthetic genes. The data provided no conclusive information (Figure 3.19). However, cc478 gave no expression level for ERG7. Erg7 is a critical enzyme in ergosterol biosynthesis in higher plants and algae as it is responsible for the conversion of squalene epoxide to cycloartenol versus lanosterol. It would be of specific interest to study whether or not this gene has any role in cooperation with other genes of the IFT machinery. ERG11 is also down-regulated in strain cc478. This data may or may not be significant, but cc478 does raise some interesting questions, due to the regulation of these two key genes, ERG7 and ERG11 during reflagellation.

3.3 Discussion

Biochemical research has provided extensive knowledge about the lipid composition of C. reinhardtii membranes, but little is known about the enzymatic mechanisms by which these lipids are produced. This chapter presents a bioinformatics analysis of the genes involved in ergosterol biosynthesis in C. reinhardtii. In a recent review by Pierre Benveniste (Benveniste, 2004), he gives a thorough analysis and
Figure 3.18. Time course analysis of ergosterol gene expression during reflagellation. The expression of each gene was set at 1 for the control cells. Error bars represent standard deviation of these replicates.
Figure 3.19. Q-RT analysis showing the difference in ergosterol gene expression between wild types cells with flagella and several bald mutants lacking flagella.
description of each gene found in *Arabidopsis thaliana* and the hallmark experiments used to analyze each specific enzyme. Comparing the material presented by Benveniste and the two pathways for sterol biosynthesis in *S. cerevisiae* and *A. thaliana*, we gathered information to construct a putative pathway in *C. reinhardtii* for further study and experimentation.

While it has been several decades since ergosterol and 7-dehydroporiferasterol were identified in the cell and flagella membranes in *C. reinhardtii*, scientists have not identified any important links between these two sterols and other cellular processes in the cell. However, our quantitative RT-PCR data does reveal significant ergosterol gene regulation during reflagellation. Even though most of the reflagellation studies look primarily at intraflagellar transport proteins, there may be other aspects of this phenomenon of reflagellation that yet to be discovered.

As the depth of research knowledge into lipid rafts, flagellar regeneration, photosynthesis and reproduction in this species continues to grow, the ergosterol pathway may prove to be of major interest in the manipulation of lipid species in *C. reinhardtii*. Sterol studies have proven to be extremely significant to the world of antifungal drug production in yeast. Targeting the ergosterol biosynthetic pathway enzymes has offered a unique approach to drug design. The genes of the sterol biosynthetic pathway are often seen to be highly conserved across species with variations in precursors and end-products. Bioinformatics analysis of the ergosterol biosynthetic pathway may allow for new innovative ways to identify key enzymes that may play dual roles in the structure and function of the cell membrane. Identification of a proposed pathway will give the background information needed to now definitively study ergosterol biosynthesis in the model system, *C. reinhardtii*.
CHAPTER 4

IDENTIFICATION OF ERG3, A GENE INVOLVED IN THE BIOSYNTHESIS OF ERGOSTEROL IN CHLAMYDOMONAS REINHARDTII

4.1 Introduction

One gene essential to ergosterol biosynthesis is ERG3. ERG3 encodes the C-5 sterol desaturase responsible for introducing a double bond at C-5 in the B ring of episterol (Osumi et al., 1979) (Figure 4.1). This enzyme is sensitive to cyanide and requires iron as well as molecular oxygen for its activity (Arthington-Skaggs et al., 1996). ERG3 in yeast is also associated with NAD(P)H-cytochrome b/ cytochrome b5 (Osumi et al., 1979). It has been previously demonstrated that ERG3 is required for the breakdown of respiratory substrates when cells are heme deficient (Smith & Parks, 1993). Deletion of ERG3 in S. cerevisiae produces haploid cells that overaccumulate the ergosterol precursor, episterol (Miller & Rampling, 1982, Parks et al., 1982). Episterol and ergosta-7, 22-dien-3-beta-ol are reported as possible substrates for the C-5 sterol desaturase (Daum et al., 1998). Cloning and sequencing of ERG3 has been reported in several model systems including Arabidopsis thaliana (Gachotte et al., 1996) and Homo sapiens (Miyazaki et al., 1999). In yeast, ERG3 is a non-essential gene except under environments of heme-deficiency (Smith & Parks, 1993). It has been suggested that Erg3 protein is a critical target in ergosterol biosynthesis (Nes & Dhanuka, 1988), and when other ergosterol biosynthetic enzymes are mutated, ERG3 expression is directly affected and regulated by these mutations (Arthington-Skaggs et al., 1996). Chlamydomonas reinhardtii, has elicited great interest as a model system in the field of lipid research, and
several membrane lipids of *C. reinhardtii* have been identified (Janero & Barrnett, 1981b, Janero & Barrnett, 1982a Janero & Barrnett, 1982b, Janero & Barrnett, 1981a, Giroud *et al.*, 1988). Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), diacylglyceryltrimethylhomoserine (DGTS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are the major lipids in the membranes of *Chlamydomonas*, but phosphatidylcholine (PC) and phosphatidylserine (PS) are absent (Giroud *et al.*, 1988). Although, PC is absent in the membranes of *C. reinhardtii*, our laboratory has confirmed that ethanolaminephosphtransferase from *C. reinhardtii* is capable of providing for synthesis of both PE and PC, apparently by methylation of PE in yeast via the Kennedy pathway (Yang *et al.*, 2004a, Yang *et al.*, 2004b). Like yeast, *Chlamydomonas reinhardtii* has been reported to have two major sterols in its plasma membrane: ergosterol and 7-dehydroporiferasterol (Gealt *et al.*, 1981). However, very little research has been done to elucidate the specific ergosterol biosynthetic pathway in *C. reinhardtii*, especially with respect to its functions and biosynthetic precursors. In this study, we begin to characterize the function of *ERG3* in *C. reinhardtii* by complementation in *S. cerevisiae* ergosterol mutants. Understanding the substrates and respective enzymes involved in this very complex pathway may give greater insight into the specific role of ergosterol in this algal species.

### 4.2 Results

#### 4.2.1 Sequence Analysis of the ERG3 Gene

A comparison of annotated genes in the *Chlamydomonas* genome to the yeast *ERG3* revealed a few genes with significant homology. The gene with the highest homology had a 21% sequence identity to the *ERG3* of yeast. The gene with the next
highest homology had a sequence identity of only 16%, and was much more similar to the sequence of ERG25 in yeast than ERG3. The Chlamydomonas gene also had a high similarity to ERG3 genes from higher plants. This gene was chosen for further analysis, and was tentatively named ERG3 (Accession No. XP_001701457).

ERG3 is a C-5 sterol desaturase that adds a double bond in the ring structure of episterol to produce ergosta-5,7,24(28)-trienol in the ergosterol biosynthetic pathway (Broach et al., 1991) (Figure 4.1). ERG3 in C. reinhardtii is located on chromosome 16 of the genome (Merchant et al., 2007). The predicted amino acid sequence of ERG3 aligns well with other C-5 sterol desaturases (Table 4.1 and Figure 4.2). The ERG3 gene in C. reinhardtii encodes a protein of 351 amino acids (Merchant et al., 2007). The cDNA is approximately 1.1 kb long and has six exons according to the JGI Chlamy Version 4.0 Genome (Merchant et al., 2007) (Figure 4.3). Like other known Erg3 proteins, the homologous gene in C. reinhardtii codes for four putative histidine metal binding domains (Miyazaki et al., 1999) (Figure 4.2). A genome database (http://genomeportal.jgi-psf.org/Chlre4/Chlre4.home.html) predicts that Erg3 protein has 3 transmembrane helices. The first helix is thought to be made up of amino acids 10-32, while the second and third helices correspond to amino acids 110-132 and 191-213 (Merchant et al., 2007, Marchler-Bauer et al., 2007), respectively (Figure 4.2).

4.2.2 Deletion of ERG3 by Homologous Recombination

While ERG3 from Chlamydomonas aligned well with putative plant ERG3 genes (Figure 4.1), its alignment with the characterized ERG3 protein of yeast showed a lesser degree of identity and similarity. To provide evidence that Chlamydomonas ERG3 functions as a C-5 desaturase, we designed experiments to complement a yeast erg3Δ mutant. To generate the null mutation in yeast, the ERG3 gene was deleted by
**Figure 4.1 Schematic diagram of the reaction catalyzed by ERG3 in yeast.** *ERG3* is responsible for introducing a double bond at the C-5 carbon (denoted by the asterisk) of the B-ring of episterol to produce ergosta-5,7,24(28)-trienol. This step is the second to last step in the biosynthetic pathway to ergosterol.
Table 4.1. Alignment scores among sterol C-5 desaturases of various organisms generated from ClustalW.

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Arabidopsis thaliana (AtERG3); Zea mays (ZmERG3); Chlamydomonas reinhardtii (CrERG3); Homo sapiens (HsERG3); Rattus norvegicus (RnERG3); Saccharomyces cerevisiae (ScERG3).
Figure 4.2. Amino acid sequence alignment of Sterol C-5 desaturase in different organisms. Arabidopsis thaliana (AtERG3) NCBI Accession Number CAA62079; Zea mays (ZmERG3) ACG38774; Chlamydomonas reinhardtii (CrERG3) XP_001701457; Homo sapiens (HsERG3) BAA33729; Rattus norvegicus (RnERG3) NP_446094; Saccharomyces cerevisiae (ScERG3) NP_013157. Conserved amino acid sequences shared by all organisms are denoted by an asterisk. The highlighted area corresponds to the putative histidine-containing metal binding domain. Dashed lines indicate gaps in the alignment. The bold, italicized font corresponds to the three putative transmembrane spanning regions of the Chlamydomonas ERG3 protein.
Figure 4.3. Schematic diagram of sterol C-5 desaturase in *C. reinhardtii* as annotated in the JGI *Chlamydomonas* Genome Version 4.0. ERG3 has six exons and five introns.

homologous recombination and replaced with the *URA3* marker in a diploid yeast strain (Figure 4.4A). PCR analysis was used to identify positive yeast recombinants that possessed the ERG3 mutation, and these isolates were sporulated to obtain haploid *erg3Δ* strains (Figure 4.4B). Yeasts lacking a functional ERG3 are viable, as the gene is non-essential under aerobic conditions (Arthington et al., 1991). In addition, yeast lacking ERG3 have an increased sensitivity to cycloheximide as previously described (Dudley et al., 2005). Haploid isolates obtained from yeast ERG3 knockouts demonstrated increased sensitivity to cycloheximide when grown under optimal conditions in rich media.

### 4.2.3 Complementation of ERG3 Mutants

To test the function of the *C. reinhardtii* ERG3 gene, complementation experiments were conducted using yeast haploid strains deficient in ERG3. Yeast *erg3Δ* strains display a marked hypersensitivity to low non-lethal levels of cycloheximide (Dudley et al., 2005). In these experiments, plasmids expressing the yeast ERG3 ORF, *Chlamydomonas* ERG3 ORF and the empty vector were transformed into the knockout
Figure 4.4. Construction of a yeast erg3Δ strain. (A) Schematic diagram of homologous recombination strategy used to delete ERG3 in *Saccharomyces cerevisiae*. PCR primers were designed to amplify the *URA3* marker gene containing the flanking regions of *ERG3*. (B) Ura+ isolates were screened by PCR to verify proper integration of *URA3* and deletion of *ERG3*. Only correctly integrated isolates would show a 459 base pair product using primers internal to *URA3* and upstream of the flanking homology. PCR was also used to verify the downstream end of flanking region homology with *URA3* (data not shown).
strains (Figure 4.5A and Table 4.2). The selectable marker *LEU2* was used to select for transformants on minimal media lacking leucine (Figure 4.5A), and Leu+ isolates were then screened for resistance to low levels of cycloheximide (Figure 4.5B). Transformants containing plasmid pDD1192 expressing the yeast *ERG3* ORF displayed normal resistance to low levels of cycloheximide (Figure 4.5B). Transforming the mutants with pDD1191, expressing the *Chlamydomonas ERG3* ORF, also showed enhanced resistance to the drug (Figure 4.5B). Mutant strains transformed with the pDD1193, empty vector, displayed very little growth on plates containing cycloheximide (Figure 4.5B). Previous work had also reported that yeast *ERG3* mutants are viable but cannot grow on non-fermentable carbon sources (Arthington *et al.*, 1991, Smith & Parks, 1993, Hemmi *et al.*, 1995). We used this phenotype as a second test of the ability of the *Chlamydomonas ERG3* to complement yeast *erg3Δ* strains. Consistent with the earlier observations, transformants expressing *Chlamydomonas* or yeast *ERG3* ORFs are able to grow on acetate as a sole carbon source, while the mutants transformed with the empty vector control cannot grow (Figure 4.5C). All strains (Table 4.3) showed positive growth on the control plates with minimal media minus leucine (Figure 4.5D).

To further characterize the degree of complementation, qualitative GC/MS analysis was performed to verify the presence of ergosterol in the complemented transformants. Cultures of *erg3Δ* yeast expressing *S. cerevisiae ERG3* or *C. reinhardtii ERG3* were grown to similar cell densities, collected, and lipids were extracted and derivatized for analyses. Selected ion monitoring for ion 363 of ergosterol was used to search the total ion chromatograph for ergosterol in the samples (Figure 4.6). When analyzing the total ion chromatograph data for specific ions of ergosterol, the knockout strain with the vector control was found to have a marked reduction in ergosterol in the
Figure 4.5. Phenotypic complementation of yeast erg3Δ strains. Known phenotypes of yeast erg3 null mutants (hypersensitivity to cycloheximide and inability to grow on acetate) were complemented by plasmids expressing either S. cerevisiae ERG3 or C. reinhardtii ERG3 cDNA. A) S. cerevisiae ADH1 promoter plasmid used to express ERG3 genes. Haploid yeast carrying the erg3Δ::URA3 allele were transformed with the designated plasmids and plated on dextrose minimal media lacking leucine (YMD minus Leu) to select for transformants. Single colony isolates were then re-streaked on minimal media lacking leucine and containing cycloheximide (0.13 µg/mL) (B) minimal media lacking leucine and containing acetate as sole carbon and energy sources (C), and on YMD lacking as overall growth control (D). Yeast mutant for ERG3 and containing the vector plasmid lacking an insert cannot grow on cycloheximide or acetate, while cells containing the plasmid expressing either yeast or Chlamydomonas ERG3 are able to grow. Each pair of plates represents erg3Δ yeast sporulated from two independently isolated diploid knockout isolates, 1+2 are DDY4259 and DDY4260 transformed with the vector only, 3+4 are DDY4261 and DDY4262, the same strains transformed with ADH1-yeast ERG3, and 5+6 are DDY4263 and DDY4264 with ADH1-Chlamydomonas ERG3. For the second panel, 7+8 are DDY4253 and DDY4254 transformed with the vector only, 9+10 are DDY4255 and DDY4256 transformed with ADH1-yeast ERG3, and 11+12 are DDY4257 and DDY4258 with ADH1-Chlamydomonas ERG3.
Table 4.2. Plasmids used in this study.

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Table 4.3. Strains used in this study.

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membranes compared to the complemented strains with the *ERG3* ORF from *C. reinhardtii* and *S. cerevisiae* (Figure 4.6). Gas chromatography data contained peaks with retention times for the ergosterol standard, DDY 4263, and DDY 4261 respectively at 11.46, 11.46, and 11.47 minutes. The mass ions coinciding with these specific retention times gives spectral data with ions 468, 363, 337, and 253 m/z, previously described as ergosterol mass ions by Griffiths *et al.* (Griffiths *et al.*, 2003). These experiments demonstrate that *ERG3* from *C. reinhardtii* can restore ergosterol biosynthesis in yeast.

### 4.3 Discussion

Ergosterol is the major sterol found in membranes of *C. reinhardtii*. However, sterol biosynthesis is not well understood in this organism. Several sterol mutant strains previously were identified in *C. reinhardtii* by selecting for resistance to the polyene antibiotic, nystatin (Bard *et al.*, 1978, Salimova *et al.*, 1999). Polyene antibiotics function by forming complexes with the sterols in the membrane and decreasing the membrane selective permeability, and thereby causing cell death (Lampen, 1966). In the past, this method of selection was used to identify ergosterol mutants. However, very little is still known about the substrate-enzyme specificity of the ergosterol biosynthetic pathway enzymes in *C. reinhardtii*. Unlike *C. reinhardtii*, several ergosterol mutants have been identified in yeast, and their phenotypes have been well documented (Osumi *et al.*, 1978). Yeast genetics allows not only the study of the function of yeast genes, but is also useful for cross-species complementation studies.

*ERG3* encodes a sterol C-5 desaturase, and belongs to the fatty acid hydroxylase superfamily. This superfamily of proteins includes C-5 sterol desaturases as well as C-4 sterol methyl oxidases (Marchler-Bauer *et al.*, 2007, Marchler-Bauer *et al.*, 2005, Marchler-Bauer & Bryant, 2004). This family of proteins possesses four putative iron-
binding domains (Miyazaki et al., 1999). Loss of ERG3 function leads to an apparent loss of ergosterol in the membrane and an increase in the closely related precursor, episterol. The replacement of ergosterol with episterol in yeast leads to increased sensitivity to cycloheximide and an inability to grow with acetate as a sole carbon source (Smith & Parks, 1993). The efficient gene replacement techniques used in Sacchromyces cerevisiae, has allowed for the creation of mutant strains in which the ERG3 gene has been replaced with URA3 (Rothstein, 1983, Wach et al., 1994). In the work described here we were able to complement S. cerevisiae ERG3 haploid knockouts with both yeast and the C. reinhardtii ERG3 ORFs. When these genes are put back into erg3Δ yeast strains, the resultant transformants show an increase of ergosterol in the membranes, increased resistance to cycloheximide and a restored ability to grow on the non-fermentable carbon source acetate.

While the Chlamydomonas ERG3 ORF does complement the mutation, it must be noted that it does not complement as completely as the yeast ERG3 gene. There are several explanations for this. Traditionally, 2 micron plasmids have been used in yeast for heterologous complementation, in order to increase the copy number of the plasmid and circumvent any issues regarding variations in protein stability, codon usage, and enzyme activity across species (Ludwig et al., 1993). This study uses a moderate copy number plasmid. Secondly, yeast and Chlamydomonas undergo two very different biochemical pathways for the production of sterols. Yeast use the traditional MVA (IPP) pathway for the synthesis of sterols, while Chlamydomonas utilizes the DOXP pathway for the synthesis of sterols (Lichtenthaler, 1999). Evolutionary differences across species allow for several variations in the ergosterol biosynthetic pathway. For example, Chlamydomonas and S. cerevisiae produce precursors to ergosterol by two independent
Figure 4.6. GC/MS data. Total ion chromatographs were analyzed to look specifically for the 100% ion of ergosterol. Mass ion 363 gives the best indication of the presence or absence of ergosterol in the samples. (A) Ergosterol standard, (B) Lipid extract from DDY4259 vector control, (C) DDY4263 expressing *C. reinhardtii ERG3*, (D) DDY4261 expressing *S. cerevisiae ERG3*. 
pathways so therefore similar enzymes can often catalyze different reactions (Gachotte et al., 1996, Lichtenthaler, 1999). Finally, ergosterol biosynthesis is a very metabolically taxing process that requires molecular oxygen, sources of energy, and optimal temperatures (Parks & Casey, 1995). Despite these potential complications, complementing the yeast ERG3 null mutation with the ERG3 gene from Chlamydomonas reinhardtii allows for the production of ergosterol, and increased survival of the cells during exposure to cycloheximide and growth on acetate.

Elucidation of the ergosterol biosynthetic pathway in Chlamydomonas reinhardtii is of great importance. There continues to be an interest in plant and algal sterols for commercial use as dietary supplements, and as components of cosmetics and pharmaceuticals (Fernandes & Cabral, 2007). For example, experimental evidence has shown that uptake of plant sterols can help to reduce cholesterol levels in humans (Moghadasian & Frohlich, 1999, Tapiero et al., 2003). The similarity of the C. reinhardtii ERG3 gene with that of higher plants indicates that the Chlamydomonas system may provide an excellent model system in which to study plant sterol biosynthesis. Better understanding of this pathway will lead to the development of directed knockout mutant strains in Chlamydomonas that may allow for overproduction of specific ergosterol precursors. Unlike crop plants, C. reinhardtii is relatively inexpensive to grow and with the new innovations in photobioreactors, large cultivation of algal lipids may begin to become more efficient. Although, this is the first work to elucidate the molecular function of any ergosterol biosynthetic enzyme in C. reinhardtii, further studies will hopefully uncover greater significance of this pathway in the unicellular algal system.
CHAPTER 5

CONCLUSIONS

5.1 Chlamydomonas reinhardtii Ergosterol Biosynthetic Genes

While research in the field of ergosterol biosynthesis in C. reinhardtii has been dormant for over a decade, the genes involved in the pathway have now been fully elucidated. Investigations of these genes have led to the discovery of two key genes, CPII and CAS1 (Chapter 3), that show similarities to the higher plant biosynthesis of sterols. Bioinformatics data presented here support the idea that C. reinhardtii produces cycloartenol as a precursor to ergosterol instead of lanosterol as seen in fungal species like S. cerevisiae. EST data have provided the support to identify whether or not these genes are expressed. In addition, many of these genes are expressed at reasonable levels as evidenced by the Q-RT PCR data presented in Figures 3.17, 3.18, and 3.19. Even though the presence of some of the genes, for example ERG9, is not strongly supported by EST data, there is no evidence to dismiss their involvement in ergosterol biosynthesis. Most of the sequences for these genes now have Accession numbers in the NCBI Pubmed Database (Table 5.1). While the development of a model pathway for ergosterol biosynthesis in C. reinhardtii was one of the major goals of this dissertation (Figure 3.2), there is still a vast amount of work left to be done in order to begin to fully characterize these genes and the enzymes for which they encode. Bioinformatics data gives valuable information about the locations and putative functions of the proteins, but sufficient experimental evidence is needed to confirm and characterize the function of each protein.

While sterol fluctuations have been implicated in the cell cycle (Janero et al., 1982), there has not been a correlation shown between reflagellation and ergosterol
biosynthesis until now. Innovations in molecular biology techniques have allowed the perfection of quality Q-RT PCR analysis of genes in *C. reinhardtii*. Several key genes (ERG3, ERG4, ERG5, ERG6, ERG7, ERG11) (Figure 3.18) in the ergosterol biosynthetic pathway do show upregulation during reflagellation lending to the hypothesis that in order to reflagellate the cell, new membranes must also be synthesized. The previously documented ergosterol mutant (Bard *et al.*, 1978), cc652, does show a lack of expression of ERG5 (Figure 3.17), which completely supports the original data from GC/MS profiles (Bard *et al.*, 1978) regarding the absence of a C22-desaturase. In addition, bald mutants also were analyzed by Q-RT PCR. This analysis, however, provided no conclusive data (Figure 3.19) from the specific sterol genes implicationed in the complete lack of flagella.

5.2 *Chlamydomonas reinhardtii* ERG3 Complements erg3Δ Yeast Mutants

The design of the working model for ergosterol biosynthesis in *C. reinhardtii*, bioinformatics analysis and Q-RT analysis of the specific genes involved gave a unique foundation by which I could then begin to analyze the genes by heterologous complementation in yeast. Sterol biosynthesis is variable among eukaryotic species. Higher plants metabolize squalene epoxide to cycloartenol to produce ergosterol as well as other sterol molecules, while yeasts metabolize squalene epoxide to lanosterol (Figure 3.7). This variation in substrate specificity and product formation can often make heterologous complementations with these genes quite difficult. The strategy for approaching this potential problem was to choose several genes in the pathway to study. *ERG3* and *ERG11* were both analyzed. While *ERG11* in yeast catalyzes the substrate lanosterol, while in higher plants the *ERG11* ortholog *CYP51G1* catalyzes the substrate obtusifoliol. Heterologous complementation was unsuccessful due to high substrate
specificity of the enzymes. Since \textit{ERG3} catalyzes the conversion of episterol to ergosta-5,7,24(28)-trienol (Figure 4.1) in yeast and higher plants, this gene was a likely choice for complementation. Several other genes of the pathway are homologous and did show some sequence similarity, but their substrate specificities varied between higher plants and yeast; the Erg3p specificity for episterol allowed the complementation to be a success.
Table 5.1. Gene names and NCBI Accession Numbers for Genes of the Ergosterol Biosynthetic Pathway in *C. reinhardtii*. Higher plant nomenclature denoted in parentheses.

<table>
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<th><em>Chlamydomonas</em> Ergosterol Gene</th>
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<tr>
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<td><em>CPI1</em></td>
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</tr>
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<td><em>ERG11 (CYP51G1)</em></td>
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<td><em>CD11</em></td>
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CHAPTER 6

FUTURE AND LONG RANGE SIGNIFICANCE OF THIS RESEARCH

Sterol biosynthesis has been studied for several decades and has been proven to be at the pinnacle of some of the most important discoveries in science and medicine. The longevity of this field of science is due in part to its complexity and interlocking nature with so many other biochemical pathways. While basic sciences research has provided us with several model systems to analyze sterols, the study of sterol biosynthesis in *C. reinhardtii* is still in its infancy. The data presented in this dissertation will hopefully open the door for future studies of sterol biosynthesis in *C. reinhardtii*. Bioinformatics analysis provides the framework by which a putative pathway could be designed for future testing. The use of heterologous complementation in yeast provided me with the ability to test the function of ERG3 in *C. reinhardtii*. Quantitative RT PCR analysis allowed a closer look at the regulation of these genes during reflagellation as well as analysis of ergosterol genes in bald, flagella-less mutant strains of *C. reinhardtii*.

In spite of the experiments presented in this dissertation, there are still many questions that have yet to be formulated and answered. Homologous recombination and use of knockout technologies are just beginning to be perfected in *C. reinhardtii*. In years to come, it may become increasingly easier to target specific genes of the ergosterol pathway and measure its affects and study phenotypic changes. Sterol biosynthesis intersects with so many other biochemical pathways, and it will be interesting in the future to manipulate genes of the ergosterol pathway in order to overproduce other phytosterols for hormone replacement therapies, natural birth control or even production
of natural dietary supplements. While it has been found that plant sterols in the presence of cholesterol precursors can put undue stress on the aortic valve (Helske et al., 2008), better understanding of the genes of the pathway in this alga, may allow us to create efficient synthetic, ingestible plant enzymes. These enzymes can be used to supplement the sterol enzymes in humans that can degrade these arterial plaques due to their substrate specificity of the cycloartenol precursors of the sterol pathway versus the more higher eukaryotic lanosterol pathway.

Another potential manipulation of this pathway could lead to use of this model system for biofuels production. While all of these future studies are quite intriguing, scientists may argue of the cost effectiveness of mass-producing Chlamydomonas. With the new wave towards producing a greener economy, I predict that in the next ten years, scientists will develop functional economical photobioreactors that will be able to generate algal biomass for fuel production with equal or lesser cost than our present technologies in petroleum deep sea drilling rigs. These photobioreactors will be located primarily in cities of high CO₂ emission, next to some of our oil refineries and factories, and in densely populated cities.

While basic sciences research has provided us with several model systems to analyze sterols, the study of sterol biosynthesis in C. reinhardtii is still in its infancy. But there is a vast interest in studying algal lipid biosynthesis for biofuels production. One major example is Botryococcus braunii. This green photosynthetic algae is known for making a vast array of hydrocarbons and methylated squalenes. Like C. reinhardtii, it too primarily synthesizes its lipids by the DOXP pathway. Understanding the genes involved in lipid biosynthesis may help to begin to identify specific precursors that can be used as biofuels or other commercial products. Basic science research has been the
foundation for some of the greatest innovations of our time. Specifically, sterol biosynthesis has been implicated in several aspects of human disease from Alzheimer’s disease to polycystic kidney disease. Because we now know the sterol components of the flagella, there may be some future interest in monitoring the movement or production of sterols in the whole cell to the flagella. GFP tagging is becoming a widespread technique in *C. reinhardtii*. Tagging ergosterol biosynthetic genes to look at activity during reflagellation may be a future experiment that may give greater detail as to whether or not these genes play a role in the regrowth of the flagella. Recent flagella research focused primarily on IFT proteins, but it is interesting to note that there are equal concentrations of sterol in the whole cell and flagella according to Gealt et al. (Gealt *et al.*, 1981). If we presume that the ER is producing sterols that are transported from the whole cell extending out to the flagella, one might agree that the sterols must be resynthesized everytime flagella regenerate. This hypothesis supports the idea that ergosterol biosynthetic genes have to turn on in order for membrane biogenesis of the flagella to occur. If the flagellum has a very similar proportion of sterols compared to the whole cell, then it would make sense that other lipid classes would be found in the flagellar membranes, but according to the literature (Gealt *et al.*, 1981) this is not the case. With the knowledge that flagella is comprised primarily of sterol and fatty acid according to Gealt *et al.* (Gealt *et al.*, 1981), there must be some unique cellular process that allows the flagellar membrane to exclude other lipid classes except sterols and fatty acids, and if this is the case, I have provided evidence that ergosterol biosynthesis plays some role in membrane biogenesis during reflagellation.

It is my hope that with new experimental technologies in *C. reinhardtii*, it will become easier to study these questions of interest. The primary challenge for researchers
in this field is the technology available to study these questions in this model system, but it is my firm belief that as newer protocols are developed and successfully tested, ergosterol biosynthetic studies in *C. reinhardtii* will arise as a very competitive field of lipid research.
REFERENCES


Mo, C., Valachovic, M. & Bard, M. 2004. The ERG28-encoded protein, Erg28p, interacts with both the sterol C-4 demethylation enzyme complex as well as the late biosynthetic protein, the C-24 sterol methyltransferase (Erg6p). *Biochim Biophys Acta* **1686**:30-6.


VITA

Kristy Marie Brumfield was born in Covington, Louisiana, in November, 1981. She obtained her high school degree in May 1999, from Opelousas Catholic School in Opelousas, Louisiana. She entered Xavier University of Louisiana where she majored in biology. In May 2003, she earned her Bachelor of Science degree. In August 2003, she started to pursue a Doctor of Philosophy degree at Louisiana State University, Department of Biological Sciences, Baton Rouge, Louisiana, USA. She studied ergosterol biosynthesis in *Chlamydomonas reinhardtii*. As a graduate student at Louisiana State University, she gained valuable training in research and teaching and has decided to pursue a career in academia. In the Fall of 2009, she will graduate with a Doctor of Philosophy degree in biochemistry. She will move on to an instructor position at Xavier University of Louisiana in New Orleans, Louisiana. She will continue to pursue her goals towards a tenure-track faculty position.