

**CHARACTERIZATION OF *PHYTOPHTHORA CAPSICI* ERG3 THROUGH
COMPLEMENTATION OF A YEAST ERG3 NULL MUTANT**

by

David S. Nesnow

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Sciences

Winter 2013

© 2013 Nesnow
All Rights Reserved

**CHARACTERIZATION OF *PHYTOPHTHORA CAPSICI* ERG3 THROUGH
COMPLEMENTATION OF A YEAST ERG3 NULL MUTANT**

by

David S. Nesnow

Approved: _____
Nicole Donofrio, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Blake Meyers, Ph.D.
Chair of the Department of Plant and Soil Sciences

Approved: _____
Mark Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
Charles G. Riordan, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

Thank you to my wife, Lori, and my son, Owen. Your support and distractions made life easier over this time. I would also like to thank my Mom and Dad and my brother Geoff for putting me on the right path and being a great family. Thank you to my committee for your advice and help through this thesis. I could not have accomplished this without your assistance. Lastly, thank you DuPont for providing me the means and time to complete this education.

TABLE OF CONTENTS

LIST OF TABLES	v
LIST OF FIGURES	vii
ABSTRACT	viii

Chapter

1	INTRODUCTION	1
1.1	<i>Phytophthora Capsici</i>	1
1.2	Sterols in <i>Phytophthora Spp</i>	2
1.3	C5 Desaturase	8
1.4	Sterol Function	11
	REFERENCES	17
2	METHODS	20
2.1	Materials	20
2.2	<i>Phytophthora Capsici</i> and <i>Saccharomyces Cerevisiae</i> Cultures	20
2.3	PCR Methods	22
2.4	Plasmids and Yeast Strains	23
2.5	Protein Expression	27
2.6	Sterol Analysis	28
	REFERENCES	32
3	RESULTS	33
3.1	<i>Phytophthora Capsici</i> Sequence and Structure Analysis	33
3.2	Sterol Pathway Sequence Analysis	34
3.3	Yeast Strains and Vectors	35
3.4	Protein Expression and Sterol Profile Analysis	35
	REFERENCES	55
4	DISCUSSION	56
4.1	Genomic and Protein Sequence	56

4.2	Protein Expression	58
4.3	Sterol Profile	59
	REFERENCES	66

Appendix

A.	GENOMIC AND PROTEIN SEQUENCES	69
B.	ADDITIONAL RESEARCH	71

LIST OF TABLES

Table 2.1:	Primer list	30
Table 3.1:	ClustalW values for a comparison of ERG3 genes	39
Table 3.2:	Plasmids and yeast strains	40
Table 3.3:	Area under the curve from ScN5 and ScN8 samples	41

LIST OF FIGURES

Figure 1.1:	Fungal sterol pathway starting with lanosterol	14
Figure 1.2:	Plant sterol pathway starting with cycloartenol.....	15
Figure 1.3:	C5 Desaturase reaction.....	16
Figure 2.1:	Vector diagrams.....	31
Figure 3.1:	Protein alignment with various ERG3 proteins.....	42
Figure 3.2:	TMHMM analysis of the <i>S. cerevisiae</i> and <i>P. capsici</i> ERG3 enzymes	44
Figure 3.3:	Alignment of <i>Arabidopsis thaliana</i> DWF5 with potential C7 reductase found in <i>P. capsici</i>	45
Figure 3.4:	Diagnostic PCR results for ScN4	46
Figure 3.5:	Diagnostic PCR for ScN2 and ScN3	47
Figure 3.6:	HPLC/MS data for ergosterol standard, ScN7, ScN1, and ScN2.....	48
Figure 3.7:	HPLC/MS data from samples of ScN1, ScN7, ScN9 and ScN10	49
Figure 3.8:	Western blot analysis of ScN5 and ScN8.....	50
Figure 3.9:	Chromatographs showing non-induced compared to induced ScN5 at different OD ₆₀₀ values	51
Figure 3.10:	Graph of the ratio of area under the curve of ergosterol divided by the area under the curve of (22E)-ergosta-7,22-dien-3Beta-ol plotted over time	52
Figure 3.11:	Rare codon graphic.....	53
Figure 3.12:	HPLC/MS of induced and non-induced ScN8	54

ABSTRACT

Phytophthora capsici is a major pest to a broad host of plants. It is a sterol auxotroph that can grow vegetatively without sterols but requires them for reproduction. Previous studies demonstrated the absence of a sterol biosynthesis pathway in *Phytophthora spp.* but observed Δ^7 sterols and $\Delta^{5,7}$ sterols converted to Δ^5 -sterols. In this study a *P. capsici* gene (*FL-PcERG3*) with homology to *Saccharomyces cerevisiae ERG3* was examined for potential C5 sterol desaturase (DES-5) activity through complementation of a yeast *erg3* null mutant. A FLAG-tagged *FL-PcERG3* was amplified out of genomic *P. capsici* DNA and expressed under either the *S. cerevisiae ERG3* promoter or under the inducible *Gall* promoter. The FLAG-tagged *FL-PcERG3* demonstrated expression in a western analysis. A HPLC/MS analysis confirmed the ergosterol pathway complementation of the yeast *erg3* null mutant by the putative *FL-PcERG3*. Chromatographs from the HPLC/MS analysis of the *FL-PcERG3* complemented yeast *erg3* null mutants had peaks at the same relative retention time and mass ($m/z = 379.5$) as ergosterol. The combination of data collected in this thesis and information from previous studies suggests the *FL-PcERG3* expresses a functional DES-5 in *P. capsici*. This is the first study to evaluate a putative *ERG3* gene from a *Phytophthora spp.* and provides evidence for an abridged sterol pathway in *Phytophthora spp.*

Chapter 1

INTRODUCTION

Phytophthora Capsici

P. capsici is a plant pathogen that infects a wide range of hosts, including the economically important lima bean, tomato, cucumber, squash, melon, pumpkin, eggplant and pepper plants. It is a soil borne heterothallic oomycete that spreads rapidly when inoculated in a field and can survive in the soil for years as an oospore. The degree of water saturation of the soil, in combination with warm temperatures, causes germination of oospores that initiate the infection. The disease is then spread rapidly through the large volume of asexual sporangia produced. Sporangia will release the mobile zoospores in the presence of water. The zoospores are negatively geotropic so they will swim against gravity. This will typically lead the zoospores from the soil or infection site to a stem or new plant tissue higher on the plant. The main route of disease spread is through water contaminated sporangia, zoospores or oospores, making water management the best practice for controlling this disease [1].

This hemi-biotroph pathogen spends most of its lifecycle on the host plant in two distinct stages. Infection and subsequent biotrophic stages take place when the sporangium, zoospore or oospore germinates on the surface of the plant. Hyphae spread and find regions to penetrate the cuticle layer. In some cases appresoria have been seen

at the infection site. Once *P. capsici* gains access to the interior tissue it will continue to colonize [2]. During the initial stages of colonization, there is no response from the host and a study on *Nicotiana benthamiana* noted haustoria pressing on the cell membrane creating a plant-pathogen interaction [3]. This suggests a suppression of the plant defense system during the infection stage. Finally the pathogen enters the necrotrophic stage, which kills the host cells and collapses tissue. After this stage, sporangia are formed and the cycle is repeated.

There are a few aspects that make *P. capsici* the ideal *Phytophthora* to evaluate biochemical pathways. As a cultured pathogen, *P. capsici* growth characteristics provide capabilities for numerous lab manipulations. The mycelium can cover a plate of V8 media in 10 days (3 days in the dark, 7 days in the light) at 25°C. These plates will also produce large quantities of sporangia which can be encysted to produce zoospores. In liquid cultures, *P. capsici* can saturate the solution overnight to create grams of mycelium. In addition to the availability of different growth stages of *P. capsici*, there is also a genome database available for comparison studies. Due to its rapid growth, high production of sporangia and zoospore, and broad host range, this *Phytophthora spp.* is ideal for examination of biochemical pathways.

Sterols in *Phytophthora Spp.*

Sterols are required in most organisms. *De novo* sterol biosynthesis occurs in a pathway that utilizes the long chain isoprenoid squalene, and after cyclization and several redox reactions results in sterol production. In animals the major sterol is cholesterol

while fungi and yeast create ergosterol [4]. The most abundant sterols in plants are sitosterol, stigmasterol or campesterol [4]. Plant and fungal sterol pathway analysis will aid in deciphering a potential C5-desaturase (DES-5) in *Phytophthora capsici*.

The plant and fungal sterol pathways convert squalene to squalene epoxide. In plants this is then converted to cycloartenol. Cycloartenol then goes through C24-methylation, C4 demethylation, cyclopropylsterol isomerization, C14-demethylation, C14-reduction and Δ^8, Δ^7 -isomerisation to become 24-methylenolophenol. At this point it either campesterol can be produced through C4-methylation, C5 desaturation, Δ^7 reduction and Δ^{24} reduction or sitosterol and stigmasterol can be produced through C28 methylation, C5 desaturation, Δ^7 reduction, Δ^{24} reduction and Δ^{22} desaturation [5].

(Figure 1)

In fungal sterol biosynthesis the squalene epoxide is converted to lanosterol through oxydosqualene cyclisation. A process of C14 methylation, C14 reduction, C4 demethylation, C24 methylation, Δ^8, Δ^7 isomerization, C5 desaturation, C22 desaturation, and Δ^{24} reduction to create ergosterol [5]. When comparing plant and yeast there are some major differences in sterol biosynthesis but each possesses several enzymes that could be used to complement the pathway in either organism

Sterols are secondary alcohols composed of 24 to 29 carbons which form a solid at room temperature. There are three 6-carbon rings which are fused *trans* in a nonlinear arrangement with a fourth 5-carbon ring. At position C3, there is usually a hydroxyl group that is eventually used for either ester or glycoside formation. A chain of carbons is also attached at the C17 position. This number of carbons varies depending on the type

of sterol. Also there are methyl groups found at the C10 and C13 positions. Saturation of the rings also varies between the different sterols. Some of the earlier sterols produced also possess a methyl group(s) at the C4 position. The sterols that have this addition in the C4 position are in the alpha orientation while the remaining groups at the C3, C10, C13 and C17 have the beta orientation [6]. The difference between the alpha and beta orientation is related to whether the methyl group is pointing up (alpha) or down (beta) in relation the plane of the ring in which they are attached.

The sterol pathway in *Phytophthora spp.* is not a well understood. They are sterol auxotrophs so the steps found in the early stages of sterol synthesis are not found in *Phytophthora spp.* They have been shown to produce squalene but lack the activity to form squalene epoxide [7, 8]. To obtain sterols, *Phytophthora* utilize elicitors, which are small proteins containing a cysteine rich hydrophilic region that bind sterols. The elicitors are secreted onto the host plant to trap and transport sterol back to the *Phytophthora* [9, 10, and 11].

Early studies with *Phytophthora* used feeding assays to determine if sterols are necessary for growth. And, if they are necessary for growth, what function they play in *Phytophthora* development. It was quickly determined that *Phytophthora spp* were able to grow vegetative structures in the absence of sterols. In 1976, Knights and Elliot showed an increase in dry weight mycelium of *Phytophthora cactorum* based on whether there are sterols available. It was also noticed in this study that Δ^5 sterols have a better uptake rate to the mycelium than Δ^7 and $\Delta^{5,7}$ sterols. And when Δ^7 or $\Delta^{5,7}$ sterols are

absorbed, they are converted to Δ^5 sterols in the mycelium. This provided the initial evidence for the preference of Δ^5 sterols in the mycelium [12].

Papers by Langcake, Hazel and Nes added to this study by providing proof that the type of sterol accumulated by a *Phytophthora spp.* plays significant roles in vegetative growth and in reproduction. Langcake studied the relationship of sterols with *Phytophthora infestans*. Langcake first evaluated the sterol profile found in potato leaves. This information allowed for direct evaluation of *P. infestans* growth when incubated with these sterols. He found when *P. infestans* was incubated in the presence of β -sitosterol, it produced the largest amount of sporangia (~33 sporangia/10 μ L) and when incubated with lanosterol, it produced the fewest amount of sporangia produced (~15 sporangia/10 μ L). Also noted was the colony diameter when grown in the presence of different sterols. Stigmasterol produced colonies approximately 59mm in diameter while lanosterol produced colonies approximately 17mm in diameter, which was not much different than when *P. infestans* was grown without sterols (~12mm). The implied lack of utilization of lanosterol was emphasized when the colony diameter stayed consistent as increased lanosterol concentrations were added to a constant stigmasterol concentration. Lastly, Langcake evaluated the ratio of sitosterol to cycloartenol in different cultivars of potato. This ratio was thought to be responsible for resistance found in different cultivars but he found instances of low levels of cycloartenol that still confirmed resistance to *P. infestans*. This showed that there was no evidence to suggest a relationship between this ratio and resistance to *P. infestans* [13].

Another paper which studied the effect sterols have on *P. infestans* was done by Hazel. This study evaluated the relationship of sterol concentrations found in leaves of resistant and sensitive cultivars of potato. When *P. infestans* was cultured on media containing sterols at the same concentrations as found in the most resistant potato cultivars, it produced the largest amount of sporangia, while the sterol concentrations from the cultivars with no resistance produced the lowest amount of sporangia. However, when they evaluated the sporangia production on the actual leaves from these cultivars, the opposite pattern was seen. These results lead to their conclusion that sterols did not create resistance to *P. infestans* in potato. It did confirm earlier work by Langcake, which claimed there was no effect on the *P. infestans* growth based on the ratio of cycloartenol to sitosterol. Hazel's work also added to Langcake's work by showing no difference in sporangia production based on this ratio [14].

Studies done by Nes et al. (1982) evaluated the vegetative and reproduction stages of *P. cactorum* based on the presence of different sterols. They demonstrated that different sterols resulted in different amount of oospores produced. Sitosterol, stigmasterol and spinasterol produced the largest number of oospores while cholesterol, lathosterol, desmosterol, ergostanol and lophenol progressively showed a reduction in the number of oospores [15].

In the most recent study conducted by Mashall et al. (2001), they evaluated the relationship of sterols from *Glycine max* on cultures of *Phytophthora sojae*. They found 10ppm sitosterol to provide the best conditions for the growth and reproduction of *P. sojae*. When C4-methyl sterol intermediates from early in the *G. max* sterol pathway

were incubated with *P. sojae*, there was no increase in growth or reproduction. Also, as cycloartenol is replaced for sitosterol, the growth of the *P. sojae* culture is reduced. Starting at a mixture of 30/70 (sitosterol/cycloartenol) and increasing the amount of cycloartenol there is no difference in growth when compared to *P. sojae* grown on sterol-free media. The oospore production also is affected by the replacement of sitosterol by cycloartenol. There is a steady decrease in oospore production until the ratio of 10/90 (sitosterol:cycloartenol) is reached. At this ratio there are no oospores produced. However, cycloartenol was not absorbed by *P. sojae* so this experiment documents the importance of a late stage sterol in the growth and reproduction of *P. sojae* and not the effect of cycloartenol [16].

From these studies on different *Phytophthora spp.* there are several key conclusions. Sterols are not required for vegetative growth but there is strong evidence that it is required for reproduction. *Phytophthora spp.* tend to accumulate Δ^5 sterols. The main reason for this is the sterols collected by the elicitors are pulled from a source containing mainly Δ^5 sterols. However, when *Phytophthora* are fed a $\Delta^{5,7}$ or Δ^7 sterol, they have been shown to convert the sterol to a Δ^5 sterol. Also, when the sterols from earlier in the plant sterol pathway are fed to *Phytophthora*, they are either not absorbed or they do not affect vegetative growth or reproduction. This suggests there is a potential C5 desaturase and C7 reductase found in *Phytophthora*.

C5 Desaturase

C5 desaturase (DES-5) has the function of catalyzing a C5 double bond into the B ring of Δ^7 -sterols (Figure 2). The resulting product of this reaction is a $\Delta^{5,7}$ sterol. The reaction cofactors are either NAD(P)H or NADH and cytochrome b [17]. It is also dependent on oxygen and inhibited by cyanide, hydrophobic metal chelators and cytochrome C. Due to the cyanide and metal chelators inhibiting the reaction, there is thought to be a di-iron cluster in the center of this enzyme [18]. In a paper by Rahier, a variety of deuterated labeled sterols were assayed in an in vitro study with *Zea mays* microsomes to evaluate how the DES-5 creates the double bond. From the data collected, there was evidence for a stepwise removal of the hydrogen at C5 and C6 on cholest-7en-3Beta-ol. Hydrogen on the C6 is removed by iron-bound oxygen which causes a carbon centered radical at C6. This leads to a disproportionation reaction which removes the C5 hydrogen and creates the double bond between C5 and C6 [19].

The major conserved feature of all DES-5 is eight histidines over three regions. Rahier originally thought the conserved histidines were the binding sites for the iron-bound oxygen that removes the hydrogen from the C6 position. To evaluate this, they substituted glutamic acid residues for the histidines. This should have increased the affinity of the iron-bound oxygen, but the addition of the glutamic acid residues caused a complete loss of activity [20]. In a different study conducted on *A. thaliana* *ERG3*, a single amino acid was changed in the DES-5 and then was complemented into an *erg3* yeast mutant to evaluate the effect each amino acid has on this enzyme. They found that when any of the histidines in the conserved histidine regions were changed to a

hydrophobic amino acid, there was complete loss of enzymatic activity. Also in this study, they found a few other conserved amino acid changes (G234A, P201A, H203L and H222L) which caused either complete loss of activity or reduced enzymatic activity. The conclusion made from these experiments was the conserved histidine regions create the correct conformation for the reaction and do not to serve as the catalytic center for the reaction [21]. The glutamic acid substitution for histines that caused loss of activity was the main supporting data for their conclusions. Irrespective of whether these conserved histidine regions have conformational responsibilities or catalytic activity, they are required in DES-5.

Saccharomyces cerevisiae erg3 mutants have been used frequently for evaluating this enzyme. These mutants are still able to grow under normal conditions and there are documented phenotypic conditions that can be used to evaluate the complementation of the pathway. Phenotypically, yeast *erg3* mutants have been shown to be sensitive to cycloheximide (0.05ug/mL), SDS (100ug/mL), hygromycin B (100ug/mL), trifluoperazine (80uM), 4-nitroquinoline-N-oxide (0.5uM), diamide (1mM), CaCl₂ (200mM) or NaCl (200mM) [22]. This increase in sensitivity to several compounds is thought to be related to a more porous cell membrane.

Mutation in *ERG3* in yeast is also associated with resistance to fluconazole (>256ppm). Since the mid-1970's triazoles, like fluconazole, have been used to control true fungal pathogens. The triazoles work by inhibiting the P-450-dependant lanosterol 14 α -demethylase which is present in true fungi but is absent from Oomycete pathogens. The inhibitor blocks the conversion of lanosterol to ergosterol [23]. Resistance has

increased around the different triazole fungicides. There also have been some studies that show that DES-5 has been associated with azole resistance in *S. cerevisiae* [24] and *C. albicans* [25]. In *Candida glabrata*, it was shown that disrupting the DES-5 enzyme did not affect azole-resistance, but it did provide azole-resistance if both the DES-5 and the C-14 demethylase (ERG11) were disrupted [23]. This is consistent with other studies that implicated resistance was due to a mutation in the DES-5 gene exerting suppression of ERG11 [25, 26, and 27].

In a 2005 paper by Mo and Bard, they determined interactions between all the enzymes from the ergosterol pathway using a split-ubiquitin membrane protein yeast two-hybrid system. From these experiments they found a complex network between all of the enzymes. The enzymes that had the most interactions were ERG 11 (lanosterol C14 demethylase), ERG25 (C4-methyloxidase), ERG27 (C3-ketoreductase) and ERG28 (scaffold protein). *ERG3* was shown to interact with ERG6 (C24-methyltransferase), ERG11, ERG25, ERG27 and ERG28. This showed *ERG3* has no direct interaction with the enzyme ERG2 which is the previous enzyme in the ergosterol biosynthetic pathway [28]. Since *P. capsici* does not have a complete sterol pathway and there are 2 putative late stage sterol pathway genes found in the *P. capsici* genome, knowing how *ERG3* interacts in a known sterol pathway will help characterize the putative late stage sterol pathway genes.

Sterol Function

The function of sterols can be categorized into two major activities. The first is a hormonal response or hormone promotion and the second is membrane function and integrity. Hormonal responses in plants are generically classified as brassicasteroids. These steroids were initially studied for their ability to increase growth in plants but now are seen to have many interactions that are essential for plant growth. Outside of cell elongation and division brassicasteroids impact membrane polarization, proton pumps, photosynthesis, stress responses and senescence. Brassicasteroids are derived from campesterol produced in the plant biosynthesis pathway [6].

Results from previous research shows there is no creation of hormonal sterols in *Phytophthora spp.* There also was a feeding study to evaluate the ability of *P. cactorum* to metabolize fucosterol to dealkyl fucosterol which has been seen in *Achlya bisexualis*. *A. bisexualis* creates steroid hormones from fucosterol to regulate reproduction and is an Oomycete like *P. cactorum*. Due to the high amounts of sterols that can be absorbed and the lack of sterol transformation in *P. cactorum*, Nes et al (1982) believe *Phytophthora* do not use the absorbed sterols to create hormones to regulate the reproductive cycle [15]. *Phytophthora* will most likely use sterols for membrane integrity.

Sterols influence membrane function and stability as free sterols, glucosides and esters. Sterol esters are created through the conjugation at the C3 hydroxyl group with a fatty acid. Plant sterol esters are formed from sterol precursors from the sterol biosynthesis pathway that reside in lipid droplets. These lipid droplets are inert storage

locations for the plant. They are also found in the cell membranes and in the soluble lipoprotein complexes [29].

Sterol glucosides are formed by conjugation of the hydroxyl bond at the C3 position with sugars. The function of these glucosides in plants is not well studied but they are known to reside in the plasma membrane, tonoplast and endoplasmic reticulum. Peng et al theorize that the sitosterol- β -glucoside in cotton is a primer for β -1,4-glucan chain elongation which is catalyzed by plant cellulose synthase (CesA) [30]. In *Phytophthora infestans* there have been 4 CesA genes identified that are crucial for infection. CesA are found mainly in the tips of appressoria (penetration structure) and when function is repressed through chemical impediment or gene silencing, the appressorial cell wall is lost and *P. infestans* infection process is impaired [31]. In addition to these CesA genes found in *P. infestans*, there has also been evidence of β -1,3-glucan microfibrils formed by *Phytophthora cinnamomi* (*in vitro*) [32].

The main function for sterols is the creation and maintenance of lipid-ordered membrane states (lipid rafts). Lipid rafts have been associated with cellular sorting, cytoskeleton reorganization, asymmetric growth and signal transduction. Sterols also play a role in maintaining the membrane in a liquid ordered state. If they are absent, the membrane will either be in a solid order (*so*) state or a liquid disorder (*ld*) state based on the temperature. In the *so* state the membrane becomes rigid and there is a loss of permeability while the *ld* state becomes fluid and has a very permeable membrane. In either *so* state or *ld* state, the membrane loses functionality. Plant membranes use

stigmasterol and sitosterol to keep a liquid order state over a large temperature range [33].

The importance of deciphering how sterols are used in *Phytophthora* is due to a combination of agricultural importance of this pathogen and fungicidal importance of the ergosterol pathway. This pathogen has a broad host range with the ability to spread rapidly. The ergosterol pathway has been a target for pesticides since the 1970's mainly through a variety of triazoles. *Phytophthora spp* are known sterol auxotroph's which require sterols for reproduction. Sterols are obtained from the host plant and transported back to the *Phytophthora* through the use of elicitors. These elicitors can retrieve a variety of sterols and some of these sterols, Δ^7 and $\Delta^{5,7}$ -sterols, have been converted to Δ^5 -sterols as shown in previous sterol feeding experiments. This sterol conversion suggests activity from a C5 desaturase and a C7-reductase, both of which are found in the late stages of the sterol pathway from plants, but no studies have been conducted to evaluate these putative sterol pathway enzymes *in vitro*. This thesis will test if *P. capsici* has a functional *ERG3* (C5 desaturase) gene which can be used to compliment the ergosterol pathway in a *Saccharomyces cerevisiae erg3* null mutant.

Figure 1.1. Fungal sterol pathway starting with lanosterol [5].

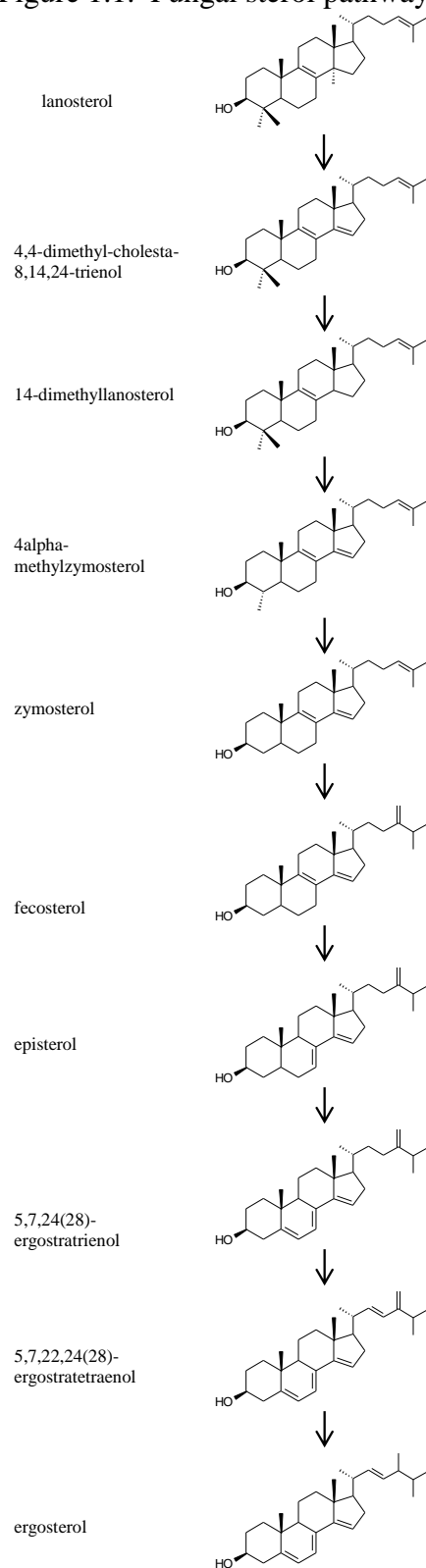


Figure 1.2. Plant sterol pathway starting with cycloartenol [5].

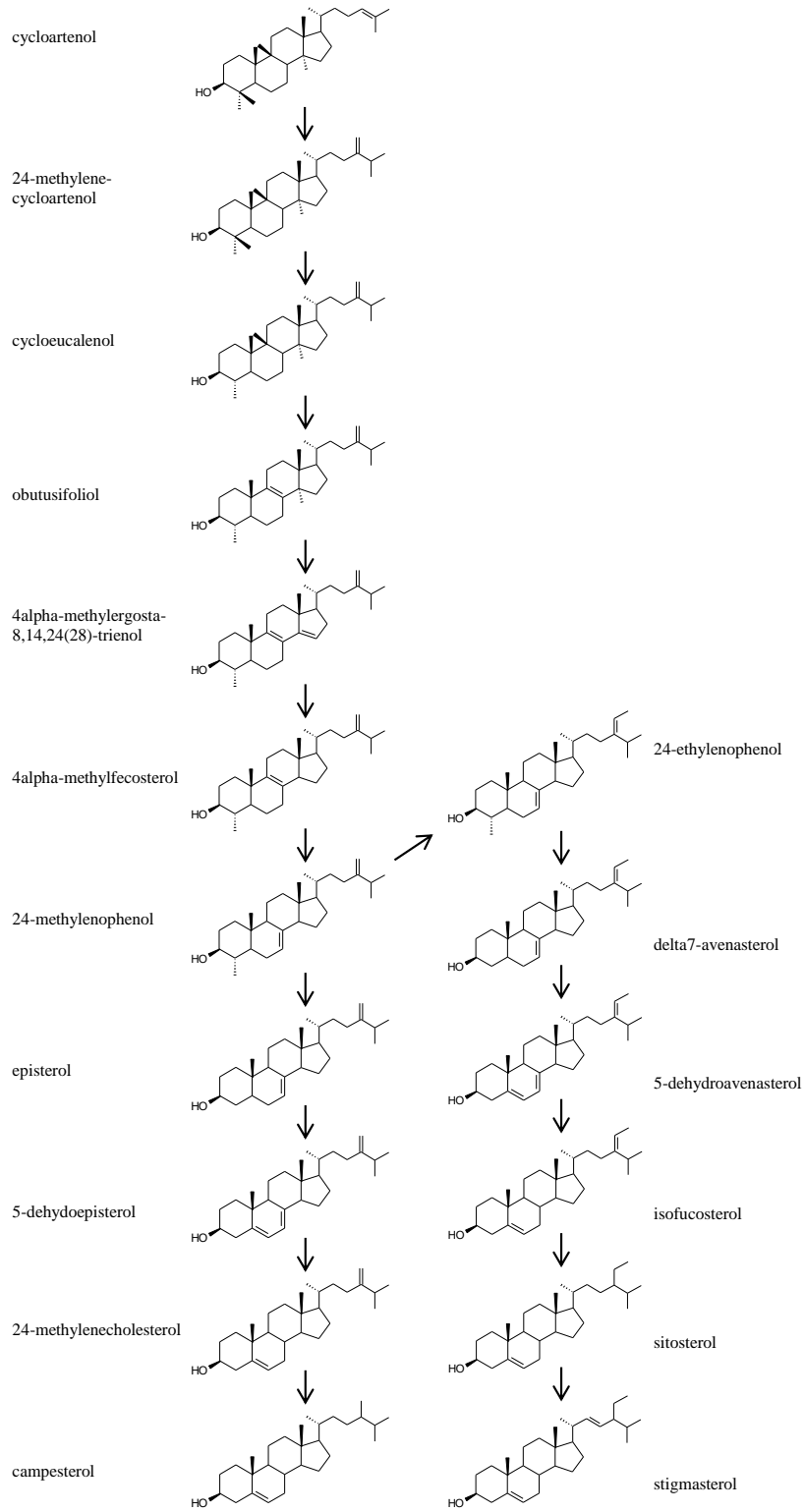
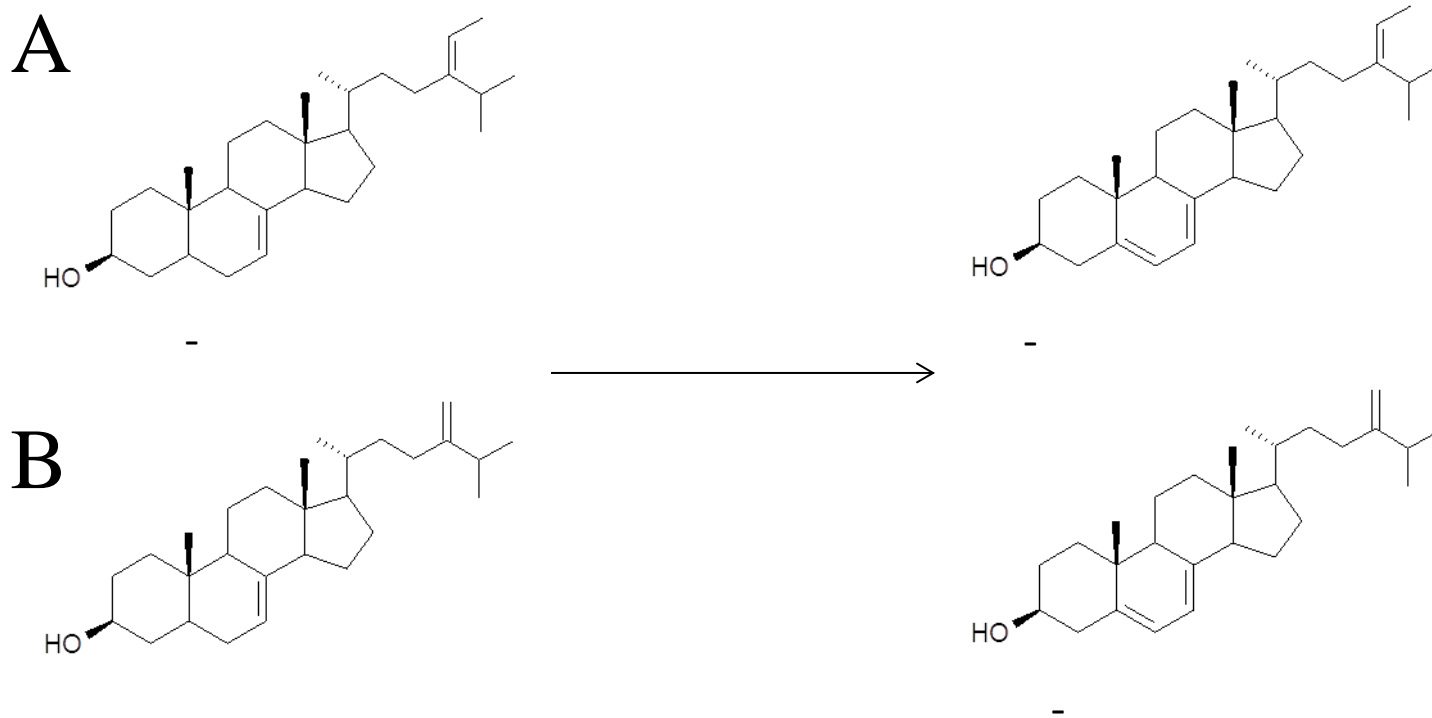


Figure 1.3. C5 desaturase reaction. A. The DES-5 reaction completed in the plant sterol pathway. B. The DES-5 reaction completed in yeast, fungi and plant.



REFERENCES

1. Hausbeck, M. K., et al. (2004). Phytophthora capsici on vegetable crops: Research progress and management challenges. *Plant Disease*. 88: 1292-1303.
2. Lamour, K. T., et al. (2012). The oomycete broad-host-range pathogen Phytophthora capsici. *Molecular Plant Pathology*. 13: 329–337.
3. Schornack, S., et al. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proc. Natl. Acad. Sci.* 107: 421–17 426.
4. Nes, W. R. (1974). Role of Sterols in Membranes, *Lipids*. 9: 596-612.
5. Desmond, E., et al. (2004). Phylogenomics of sterol synthesis: Insights into the origin, evolution, and diversity of a key eukaryotic feature. *Genome Biology and Evolution*. 1: 364-381.
6. Grunwald, C. (1978). Function of sterols. *Phil. Trans. R. Soc. Lond. B*. 284: 541-558.
7. Gottlieb, D., et al. (1978). Differences in the sterol synthesizing pathways of sterol producing and non-sterol producing fungi. *Phytopathology*. 68: 1168-1169.
8. Wood, S.G., et al. (1978). Evidence from cell-free systems for differences in the sterol biosynthetic pathway of Rhizoctonia solani and Phytophthora cinnamomi. *Biochem. J.* 170: 355-363.
9. Mikes, V., et al. (1997). The fungal elicitor cryptogein is a sterol carrier protein. *FEBS Letters*. 416: 190–192.
10. Vauthrin, S., et al. (1999). Elicitins traps and transfer sterols from micelles, liposomes, and plant plasma membranes. *Biochimica et Biophysica Acta*. 1419: 335–342.
11. Osman, H., et al. (2001). Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. *Molecular Biology of the Cell*. 12: 2825–2834.

12. Knights, B. A. et al. (1976). Metabolism of Δ^7 and $\Delta^{5,7}$ sterols by *Phytophthora cactorum*. *Biochimica et Biophysica Acta*. 441: 341-346.
13. Langcake, P. (1974). Sterols in potato leaves and their effects on growth and sporulation of *Phytophthora infestans*. *Trans. Br. mycol. Soc.* 6: 573-586.
14. Hazel, W. J. (1988). Relationship of potato leaf sterols to development of potato leaf blight caused by *Phytophthora infestans* on U.S. potato clones and breeding lines. *Plant Disease*. 72: 203-205.
15. Nes, W. D., et al. (1982). Role of steroids and triterpenoids in the growth and reproduction of *Phytophthora cactorum*. *Lipids*. 17: 178-183.
16. Marshall, J.A., et al. (2001). Soybean sterol composition and utilization by *Phytophthora sojae*. *Phytochemistry*. 58: 423-428.
17. Rahier, A., et al. (1997). The Role of Cytochrome b5 in 4 α -Methyl-Oxidation and C5(6) Desaturation of Plant Sterol Precursors. *Biochemical and biophysical research communications*. 236: 434-437.
18. Shanklin, J., et al. (1997). Mossbauer studies of alkane ν -hydroxylase: Evidence for a diiron cluster in an integral-membrane enzyme. *Proc. Natl. Acad. Sci U.S.A.* 94: 2981-2986.
19. Rahier, A. (2001). Deuterated Δ^7 -Cholestenol Analogues as Mechanistic Probes for Wild-Type and Mutated Δ^7 -Sterol-C5(6)-desaturase. *Biochemistry* 40: 256-267.
20. Rahier, A., et al. (2000). Biochemistry and site-directed mutational analysis of Δ^7 -sterol-C(6)5-desaturase. *Biochemical Society Transactions*. 28: 799-803.
21. Taton, M., et al. (2000). Role of Highly Conserved Residues in the Reaction Catalyzed by Recombinant Δ^7 -Sterol-C5(6)-Desaturase Studied by Site-Directed Mutagenesis. *Biochemistry*. 39: 701-711.
22. Hemmi, K., et al. (1995). The physiological roles of membrane ergosterol as revealed by the phenotypes of *syr1/erg3* null mutants of *Saccharomyces cerevisiae*. *Biosci. Biotech. Biochem.* 59: 482-486.
23. Geber, A., et al. (1995). Deletion of the *Candida glabrata* ERG3 and ERG11 Genes: Effect on cell viability, Cell growth, sterol composition, and antifungal susceptibility. *Antimicrobial Agents and Chemotherapy*. 39: 2708-2717.

24. Watson, P. F., et al. (1988). Isolation and analysis of ketoconazole mutants of *Saccharomyces cerevisiae*. *J. Med. Vet. Mycol.* 26:153-162.
25. Howell, S. A., et al. (1990). A comparison of the sterol content of the *Candida albicans* Darlington strain with other clinically azole-sensitive and -resistant strains. *J. Appl. Bacteriol.* 69: 692-696.
26. Bard, M., et al. (1993). Sterol synthesis and viability of *erg11* (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. *Lipids* 28: 963-967.
27. Taylor, F. R., et al. (1983). Requirement for a second sterol biosynthetic mutation for viability of a sterol C-14 demethylation defect in *Saccharomyces cerevisiae*. *J. Bacteriol.* 155: 64-68.
28. Mo C., et al. (2005). Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex. *J. Lipid Res.* 46: 1991-1998.
29. Dyas, L., et al. (1993). Steryl fatty acyl esters in plants. *Phytochemistry*, 34: 17-29.
30. Peng, L., et al. (2002). Sitosterol- β -glucoside as Primer for Cellulose Synthesis in Plants. *Science* 295: 147-150.
31. Grenville-Briggs, L. A., et al. (2008). Cellulose Synthesis in *Phytophthora infestans* is required for normal appressorium formation and successful infection of potato. *The Plant Cell.* 20: 720-738.
32. Wang, M. C., et al. (1976). Synthesis of β -1,3-Glucan microfibrils by a cell-free extract from *Phytophthora cinnamomi*. *Archives of biochemistry and biophysics.* 175: 351-354.
33. Dufourc, E. J. (2008). Sterols and membrane dynamics. *J Chem Biol.* 1: 63-77.

Chapter 2

METHODS

Materials

Growth media was purchased from VWR Scientific and chemicals were purchased from Sigma-Aldrich. V8 juice was provided by DuPont. Plasmids were generously provided by James A. Sweigard (DuPont, Crop Protection). Yeast strains were purchased from ATCC and Top10 competent bacterial cells were purchased from Invitrogen. Phusion polymerase and DNA restriction enzymes were purchased from New England Biolabs. High Pressure Liquid Chromatography (HPLC) grade water and methanol was purchased from EMD Chemicals.

***Phytophthora Capsici* and *Saccharomyces Cerevisiae* Cultures**

A *P. capsici* culture was provided by J. A. Sweigard (DuPont, Crop Protection). This culture is a single spore isolate from a University of Delaware culture. Encysted zoospores were grown on V-8 plates (200mL/L V8 juice, 4.5g/L CaCO₃, 20g/L agar) at 25°C for 2 days in the dark. The plates were then moved to a light 25°C chamber and allowed to grow for an additional 7 days. After 7 days, the plates were placed in a 4°C chamber for 30 minutes to start the release of zoospores. The plates were then removed from the 4°C chamber and sterile water was added to the plates (~5 mL). Only enough

water to cover the mycelium was added to each plate. The plates were incubated at room temperature (RT) for 30 minutes. The water/spore mixture was then decanted from the plates and placed into a sterile glass beaker. An equal volume of sterile encystment fluid (20g/L Tryptone, 5g/L Yeast Extract, 5mM CaCl₂-2H₂O, 20mM Glucose, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂-6H₂O, and 10mM MgSO₄-7H₂O) was added to the zoospore mixture. After 30 minutes, the inoculum was then added to 0.5L of Malt+ growth media (20g/L Malt Extract, 4g/L Peptone and 4g/L Yeast Extract) in a 2L baffled flask. The culture was grown at 27.5°C overnight at 100rpm in a New Brunswick Scientific innova 44 incubator. To extract the *P. capsici* genomic DNA (gPC), 2mL of the O/N culture was centrifuged in a Thermo Scientific Heraeus Pico 17 centrifuge at max speed for 3 minutes. The media was removed and 300µL of DNA extraction buffer (100mM TRIS pH7.4, 100mM NaCl, 10mM EDTA and 0.1% v/v Triton X-100) was used to re-suspend the pellet. This was then transferred to a tube used for bead beating. The cells were disrupted for 30 seconds in a Biospec Products Mini-Beadbeater and then incubated at 70°C for 10 minutes. The tube was then centrifuged at max speed for 2 minutes and the supernatant containing the gPC-DNA was collected.

Two strains, Sc64 ((ATCC #4012667, MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 Δ ERG3) and Sc69 (ATCC #208277, MAT α prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2), of yeast were grown in 2mL of sterile YPD media (10g/L Yeast Extract, 20g/L Peptone and 20g/L glucose) to an OD₆₀₀ of 3 in 14mL BD Falcon round bottom tubes on a Bellco Biotechnology Cell Production Roller Drum at 30°C in the dark [1, 2, 3]. To extract genomic DNA from yeast, the cells were then transferred to a 2mL Eppendorf

tube and centrifuged at 17000 x g for 1 minute. The supernatant was removed and the pellets were collected. Genomic DNA for the two strains (gSc64 and gSc69) was isolated and collected following the same protocol used for the gPC.

PCR Method

PCR reactions were conducted on an Applied Biosystems GeneAmp PCR system 9700. Each reaction was 50 μ L and consisted of 47.5 μ L NEB GC buffer, 0.5 μ L NEB Phusion Polymerase, 0.5 μ L 100uM Primer A, 0.5 μ L 100uM Primer B and 1 μ L of DNA. The PCR temperature program follows the protocol given by NEB with variations in the T_m values and extension times based on primer and gene size. Every PCR reaction and plasmid purification was desalted with Qiagen PCR cleanup kits and with Qiagen MiniPrep kits, respectively. All oligonucleotide primers were purchased from Sigma Aldrich and diluted to 100uM in Fluka TRIS-EDTA buffer (Table 1). Genomic DNA sample for electrophoresis were prepared by mixing 18 μ L of DNA with 2 μ L 10x Loading Dye (0.25M EDTA, 50% Glycerol, 0.25 % Bromophenol Blue and 0.25% Xylene Cyanol). This was loaded onto 1% agarose + TBE (10.8g/L TRIS base, 5.5g/L Boric acid and 0.93g/L EDTA) + 0.25ug/mL Ethidium Bromide gels and run at 110V. Gels were visualized on a Fotodyne UV box and photos were taken using a Polaroid camera with Fuji Film FP-3000B.

Plasmids and Yeast Strains

A protease deficient yeast strain (Sc69) was used to reduce the possibility of the *P. capsici* *ERG3* protein product, C5 desaturase (DES-5), being degraded. Sc64 is an *erg3*- mutant from the systematic yeast knock-out collection. The genomic DNA of Sc64 therefore contains the *kanMX* marker at the *Erg3* locus which can be used as a source of *erg3::kanMX* DNA. To obtain *erg3::kanMX*, a PCR reaction using genomic DNA from ATCC yeast *erg3*, gSc64, with primers 1573 and 1574 amplified the *kanMX* gene with 300bp of Sc69 *ERG3* promoter and terminator regions flanking each end of the gene. This construct was used to knockout the yeast *ERG3* gene in Sc69. The *kanMX::erg3* fragment was transformed into Sc69 using the lithium acetate method [4]. Sc69 was grown overnight at 30°C in YPD media on a drum shaker. This starter culture was diluted in YPD to make a 50mL culture at a cell density of 1×10^5 . The cell concentration was determined by counting the cells on a glass hemocytometer. After 2-3 divisions, the yeast were centrifuged at 1000 x g for 5 minutes and the supernatant was decanted. The pellet was re-suspended in 25mL sterile water and centrifuged again. The supernatant was decanted and the pelleted was re-suspended in 1mL 100mM lithium acetate. This was centrifuged at 15000 x g for 15 seconds and the supernatant was removed. The pellet was then re-suspended in 400μL of 100mM lithium acetate to create the competent yeast cells. 50μL of the competent yeast cells was used for each transformation. Competent yeast cells were transformed by combining, 240μL of 50% PEG, 36μL of 1M lithium acetate, 2.5μL of boiled Herring sperm DNA, and 50μL of sterile water with 22.5μL PCR product. This mixture was incubated in a 30°C water bath

for 30 minutes and then transferred to a 42°C water bath for 40 minutes. Transformed cells were then plated onto a 2% agar YPD + 200ppm Geneticin plate and the protease deficient *erg3* knockout (ScN4) was confirmed through diagnostic PCR reactions, sequencing and sterol profile analysis.

The Pc *ERG3* gene was found in the *P. capsici* genome database after running a BLASTp with the *S. cerevisiae* *ERG3* protein sequence. This provided the majority of the predicted gene, but after evaluating the sequence with FGENESH (<http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=PhycaF7&id=75302>), it was thought that this gene consisted of 3 exons. To produce the full gene, a PCR reaction using gPC and the primers 1839 and 1840 with a T_m of 50°C and an extension time of 1 minute was performed. This PCR product was ligated into pRS425 (LEU2) following the protocol given by NEB for their T4 ligase. The ligation product was used to transform Invitrogen Top10 cells following the manufactures protocol. The transformed bacteria was plated onto plates of 1% agar Difco LB broth + carbenocillin (100ug/mL) plates and incubated overnight at 37°C. Colonies were grown in 2mL LB + 100ppm carbenocillin overnight at 37°C and plasmid DNA was purified using Qiagen Miniprep spin columns. A sample of each of the plasmids was then digested for 30 minutes at 37°C with *XbaI* and *NdeI* following the buffer system and protocol given by NEB. The plasmids that had the appropriately sized inserts were then analyzed by the DuPont Sequencing Lab. The correct plasmid was used in a PCR reaction with primers 2753 and 2754 at a T_m of 51°C and an extension time of 1.5 minutes. This provided the putative full length *P. capsici*

ERG3 gene (*FL-PcERG3*). The same cloning procedures were followed to obtain the full *FL-PcERG3* gene in plasmid pRS425 (pJS495).

The initial promoter used was 300bp of the Sc69 *ERG3* promoter. This was amplified with primers 1983 and 1984 from gSc69 at a T_m of 50°C and an extension time of 30 seconds. A three way ligation was performed with this *NotI/NdeI* digested amplified product, *NdeI/XbaI* digested *FL-PcERG3* and *NotI/XbaI* digested pRS425 to create plasmid pDN1. The Sc69 *ERG3* was amplified using primers 1601 and 1602 at a T_m of 49°C and an extension time of 1 minute. This was ligated into a *NotI/XhoI* digested pRS425 (pDN2). A 1xFLAG-tag was added to 3` end of *FL-PcERG3* by amplifying with primers 1839 and N18 at a T_m of 50°C and an extension time of 1 minute. This was digested with *NdeI* and *XbaI* and ligated in *NdeI/XbaI* digested pDN1 (pDN3). A 1xFLAG-tag was added to 3` end of Sc69 *ERG3* by amplifying with primers 1601 and N15 at a T_m of 50°C and an extension time of 1 minute. This was digested with *NotI* and ligated in *NotI* digested pRS425 (pDN4).

Two PCR amplifications were needed to add a 1xFLAG tag to the 3` end of the *FL-PcERG3* and the Sc69 *ERG3*. These amplifications allowed ligation into both pRS425 and pJS467. The first reaction for the *FL-PcERG3* gene was done with primers N18 and N19 and the second reaction was done with N18 and N20 (Table 1). The first reaction for the Sc69 *ERG3* gene was completed with primers N13 and N15 and the second reaction was done with N14 and N15. Both reactions for the *PcERG3* gene and the Sc69 *ERG3* gene used a T_m of 50°C and an extension time of 1.5 minutes. The *FL-PcERG3* used pJS495 DNA and the Sc69 *ERG3* used gSc69 DNA. 20µL of each

desalted PCR product was then digested with *NotI*. The digests for the *FL-PcERG3* gene were mixed together and the digests for the Sc69 *ERG3* were mixed together. These mixtures were annealed at 98°C for 3 minutes and then allowed to cool to room temperature before being used in a ligation. Ligations were done with digested PCR product and *NcoI/NotI* digested pJS467 (pDN5 and pDN6).

Due to the extremely poor codon selection in the *FL-Pc ERG3* gene, the sequence was sent to GenScript to synthesize a yeast optimized version of the gene. This was designed to have *NcoI* and *BamHI* restriction enzyme sites for a ligation into plasmid pJS564. This gene came in vector pUC57 and was digested and ligated into pJS564 following protocols previously described above (pDN7).

After plasmids were sequenced (Figure 1), they were transformed into ScN4. This followed the same lithium acetate transformation procedure as previously described. Also, ScN4 and Sc69 were transformed pRS425 (-Leu) to have positive and negative control yeast strains, ScN1 and ScN7. Colonies from each of the transformations were selected and grown in 2mL selective growth media (6.7g/L Yeast Nitrogen Base w/o amino acids, 2g/L Drop Out – Leucine or Tryptophane, 20g/L Raffinose and 5mL 1N NaOH). These were grown for 2 days at 30°C in a Bellco Biotechnology Cell Production Roller Drum and were used to start 250mL cultures at an OD₆₀₀ of 0.02 in either selective growth media or induction media (selective growth media + 0.111M Galactose). These were grown over 4 days and a 50 mL sample was taken for each growth condition for each strain on each day. The samples taken were eventually used for sterol analysis, protein extraction and cell counts. The samples were centrifuged at 1000xg for 5

minutes, supernatants decanted and the cells were then washed in 25mL PBS buffer (1.7mM KH_2PO_4 , 136.8mM NaCl, 2.7mM KCl, 10.1mM Na_2HPO_4) three times. After the third wash, cells were centrifuged at 1000xg, supernatants were removed and the cell pellets were frozen in liquid N_2 and stored at -80°C .

Protein Expression

Protein expression was evaluated using Western blot assays. Protein for these assays were collected by first re-suspending the yeast cells in 1mL of extraction buffer (50mM HEPES 7.5, 50mM NaCl, 2mM Dithiothreitol, 1ug/mL Roche Protease Inhibitor cocktail and 5% glycerol). From this 1mL, 300 μL was pipetted into a 2mL screw-top tube containing approximately 200 μL volume of glass beads. During this process, the tubes were kept on ice. Once all the cell samples were assembled, they were beat in a bead beater for 6 x 30 seconds. In between beatings, the tubes were kept on ice for 1.5 minutes. The samples were then centrifuged at 12,000 x g for 2 minutes at room temperature. The supernatants were collected and protein concentrations were determined using a Bradford Protein Assay [5] and analyzed with a Hewlett Packard Diode Array Spectrophotometer at an absorbance of 595nm. Twenty micrograms of protein from each sample was mixed with 1x Invitrogen LDS loading dye and reducing agent. These were then loaded onto Invitrogen NuPAGE Novex 4-16% Bis-Tris gels for SDS-PAGE. After SDS-PAGE was complete, gels were placed in Invitrolon PVDF/paper sandwiches and then blotted onto PVDF membrane with Invitrogen XCell II

blot system. The transfer process was run at 30V for 2 hours following the setup and protocol provided by Invitrogen.

The blotted PVDF membranes were blocked overnight at 4°C in 1x PBS, 0.1% Tween 20 (v/v) and 5% (w/v) Skim Milk on a rotary shaker. After overnight incubation, the membranes were washed twice in washing buffer (1x PBS and 0.1% Tween 20) and then placed into fresh washing buffer for 15 minutes on an orbital shaker. The wash cycle was completed with two 5 minute washes. Primary antibody, Sigma Aldrich monoclonal ANTI-FLAG M2-peroxidase (Horseradish Peroxidase) antibody from mouse, was added to blocking buffer at 0.125ng/mL. Fifty mL of the primary antibody mixture was incubated with each of the membranes for 2 hours at RT on an orbital shaker. The membranes were washed as described before and then incubated with 50mL of the secondary antibody, 0.1ng/mL polyclonal Donkey anti-Mouse IgG (HRP), for 1 hour at RT on an orbital shaker. Blots were then washed as describe above and then incubated with Novex ECL Chemiluminescent Substrate to produce a chemiluminescent signal on the blots. The blots were exposed to GE Amersham Hyperfilm ECL in the dark and films were developed in an AFP Imaging Mini-Medical developer.

Sterol Analysis

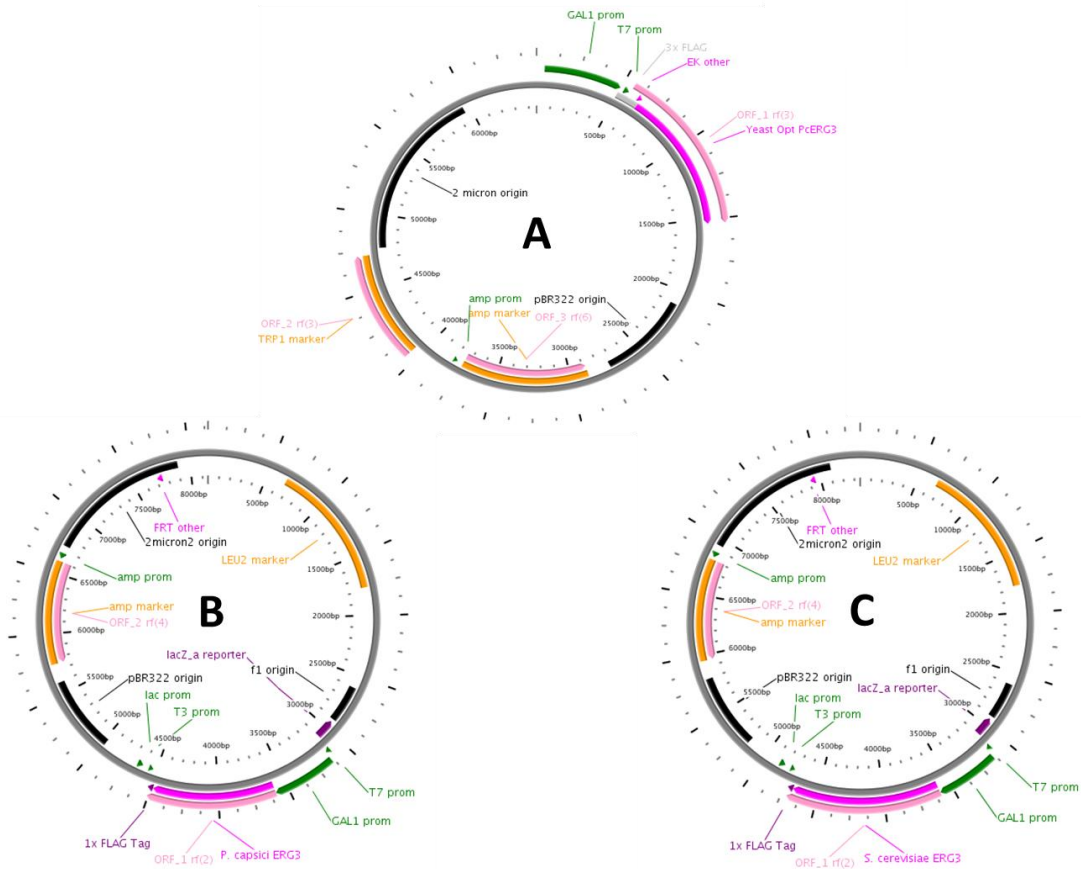
The ability of expressed *FL-PcERG3* to convert episterol to 5,7,24(28)-ergostatrienol was tested on an Agilent 1100 series HPLC and masses identified using an in-line Finnigan LTQ mass spectrometer equipped with an APCI source (Atmospheric-pressure chemical ionization). The collected yeast cells were re-suspended in 300µL

HPLC grade methanol and were bead beat for 1 minute. This solution was centrifuged for 2 minutes at 12,000 x g at RT. The supernatants were removed and filtered through a 0.2um filter to remove any debris. They were then loaded into auto-sample vials and then 10µL was injected for HPLC/MS analysis. The LC was performed on a 4.6mm x 20mm Waters Atlantis dc18 IS column (5µm beads) at 2mL/minute. The buffer system employed was 0.1% formic acid (FA) and methanol. The initial gradient started at 40% FA/60% methanol and linearly increased to 5% FA/95% methanol over 3 minutes. The 5% FA/95% remained constant for 3.5 minutes and then was re-equilibrated with start buffer (40% FA/60%) to prepare for the next sample. Ergosterol was used as a standard to establish the relative retention time and m/z for identification of this sterol end product. The mass spectra data was analyzed using the Qual Browser module of the Excalibur control software for the LTQ mass spectrometer.

Table 2.1. Primer list. This table shows the name of the primer, the sequence, the nearest neighbor Tm and a brief description. Restriction enzyme sequences are underlined and bold. FLAG-tag sequence is in Dark Blue.

Name	Sequence	Tm	Comments
1573	CGAAAAATTTCAGGCTCGTAT	50	Sc69 ERG3 -300 from ATG
1574	AGTTAACACTTGCGCTTCA	50	Sc69 ERG3 +300 from stop codon
1618	GGCCATTCTAAACTATACATGAAGAT	53	Sc69 ERG3 486bp from start_forward primer
1839	TGTCG CATATG ATGGACCTCATCCTGGAATA	50	FL-PcERG3 5' Forward w/NdeI
1840	TGTCG TCTAGA GTAGGATCCAAACATTCGGT	49	FL-PcERG3 3' Reverse w/XbaI
1983	TGTCG CTCGAG GGCATTATTCGGTCGTTTA	49	Sc69 Promoter Forward w/XhoI
1984	TGTCG CATATG ATCTCAAATCTAGACGAATATTTTT	50	Sc69 Promoter Reverse w/NdeI
2753	TCTAGACT ATGTTTCCTTGGGTATGGAGTAACCTGTGGTAGGTGGTTCGCGGTAGGATCCAAACATTCGGTC	50	FL-PcERG3 reverse 3' extra amino acids & XbaI
2754	CATATG AGATTGTGGAACACTTCAGCGCCATCGCGCTGCATGACAATCCGAG	51	FL-PcERG3 forward 5' extra amino acids & NdeI
2831	CGATTAATTCCAACATGGATG	50	KanMX +33 from ATG Forward
2832	AGCCATTACGCTCGTCA	50	KanMX +494 from ATG Reverse
N13	G ATTTGGTCTTAGAAGTCGCT	50	Sc69 ERG3 5'a for NcoI compatible end
N14	CATGG ATTTGGTCTTAGAAGTC	50	Sc69 ERG3 5'b for NcoI compatible end
N15	CATTAACC GCGGCCGC CCTA ATAATCATCATCTTTATAATCT CAGTTGTTCTTCTTGGTATTTG	50	Sc69 ERG3 3' with FLAG and NotI
N18	CATTAACC GCGGCCGC CCTA ATAATCATCATCTTTATAATCT GTTTCCTTGGGTATGGAGTA	50	FL-PcERG3 3' with FLAG and NotI
N19	AGATTGTGGAACACTTCAGC	50	FL-PcERG3 5'a for NcoI compatible end
N20	CATG AGATTGTGGAACACTTC	49	FL-PcERG3 5'b for NcoI compatible end
N23	TGTCG CCATGG ATTATAAAGATGATGATGATTATATGAGATTGTGGAACACTTC	51	Pc FLAG 5' NcoI
N24	TGTCG GCGGCCGC TATGTTTCCTTGGGTATGGAGTAA	51	FL-PcERG3 Stop NotI
N27	TGTCG GCGGCCGC TAGTTGTTCTTCTTGGTATTTGGGT	51	Sc69 ERG3 Stop NotI

Figure 2.1. Vector diagrams. These diagrams show the major features included in each of the vectors used in this study. Vector A shows the 5' 3xFLAG-tagged *FL-PcERG3* gene in pJS564. Vectors B and C illustrate the *FL-PcERG3* and *S. cerevisiae ERG3*, respectively, in the vector pRS425. Both B and C have 1xFLAG-tags at their 3' end. All inserts are under a Gal1 promoter.



REFERENCES

1. Winzeler E.A., et al. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285: 901-906, 1999.
2. Zubenko GS, et al. (1980). Mapping of the proteinase b structural gene PRB1, in *Saccharomyces cerevisiae* and identification of nonsense alleles within the locus. *Genetics* 96: 137-146, 1980.
3. Jones EW. (1977). Proteinase mutants of *Saccharomyces cerevisiae*. *Genetics* 85: 23-33, 1977.
4. Gietz, R.D., et al. (2002). Transformation of yeast by the Liac/SS carrier DNA/PEG method. *Meth.Enzymol.* 350: 87-96. 2002.
5. Bradford, M.M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254, 1976.

Chapter 3

RESULTS

Phytophthora Capsici *ERG3* Sequence and Structure Analysis

A BLASTN analysis of the *FL-PcERG3* gene compared to the yeast genome database revealed 60% identity with 5% gaps while the Yeast Optimized *FL-PcERG3* gene has 62% identity with 6% gaps [1]. A BLASTP of the *P. capsici* DES-5 sequence has 46% identity, 62% positives with 1% gaps. Table 1 shows the scores from a ClustalW protein alignment of *ERG3* from *P. capsici*, *S. cerevisiae*, *Phytophthora sojae*, *Homo sapiens*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *Chlamydomonas reinhardtii*. Each of the putative *ERG3* genes listed, excluding the *P. sojae* and *P. capsici* *ERG3*, have been transformed into a yeast *erg3* KO mutant and have complemented the ergosterol pathway [2, 3, 4, 5, 6].

There are conserved histidines found in all C-5 Sterol desaturase enzymes. When a variety of these proteins are aligned using ClustalW, the *P. capsici* DES-5 has the 3 conserved histidine regions seen in all C5-desaturase enzymes. In addition to the conserved histidine regions, there are single amino acid changes that have been documented in the *A. thaliana* and *Zea Mays* *ERG3* enzymes that have caused either significantly reduced enzyme activity or complete loss of enzyme activity. None of these amino acid changes were found in the *P. capsici* DES-5 sequence when aligned with *A. thaliana* (Figure 1).

The transmembrane prediction tool TMHMM [7], predicted 4 transmembrane helices in the *P. capsici* DES-5 and 3 transmembrane helices in the *S. cerevisiae* DES-5. There is potentially an additional transmembrane helix in the *S. cerevisiae* DES-5 prediction, but it is below the default TMHMM probability score (Figure 2). UniProt (<http://www.uniprot.org/uniprot/P32353>) also predicts *S. cerevisiae* DES-5 to have 3 transmembrane helices, but removes the 181-203 helix that is predicted by TMHMM and instead predicts one from 243-263 (Figure 1).

Sterol Pathway Sequence Analysis

The cofactor for the ERG3 reaction is cytochrome b5 (CTB5). This cytochrome has not been studied in the *Phytophthora spp.* but has been shown to be an important part of this reaction in other organisms. When the hypothetical *Phytophthora capsici* CTB5 amino acid sequence is analyzed through BLAST with the *Saccharomyces cerevisiae* genome, there is 86% coverage with 38% identity and 56% similarity. When it is compared with the *Arabidopsis thaliana* proteome, the best score retrieved is from cytochrome b5 isoform D. This *Arabidopsis* CTB5 has 93% coverage with 42% identities and 63% positives with 6% gaps. *P. capsici* CTB5 also has one predicted transmembrane helix from 122 to 144 and when aligned with the *A. thaliana* CBT5 using ClustalW, has the histidine amino acids that are heme axial ligands.

In addition to the ERG3 gene studied in this paper, there is potentially an additional sterol pathway gene (DWF5) that is similar in protein sequence to a gene found in *P. capsici*. This enzyme may be important to the functionality of the PcERG3 depending on specificity for the sterol substrate used in the reaction. DWF5 is a Δ^7 sterol reductase which is found in *Arabidopsis thaliana*. When *P. capsici* putative DWF5 and the *A. thaliana* DWF5 protein sequences are

aligned in BLASTP, they have 54% identity and 66% similarity with 94% coverage and 2% gaps (Figure 3). *A. thaliana* DWF5 is predicted to have 9 transmembrane helices while the potential *P. capsici* DWF5 has 6 transmembrane helices predicted by TMHMM. There are three additional potential transmembrane helices that are below the cutoff for the *P. capsici* DWF5. A BLASTP of the *P. capsici* DWF5 also predicts that it contains the conserved ERG4_ERG24 domain that is found in all C7-reductase proteins.

Yeast Strains and Vectors

ScN1 was created by replacing the yeast ERG3 gene with the KanMX selectable marker [3]. The regions flanking the KanMX marker had 300bp of homology before and after the yeast ERG3 gene. After transforming the KanMX marker into Sc69, the colonies that formed on the 200ppm geneticin selection plates were screened for the appropriate marker through the use of PCR (Figure 4). This diagnostic PCR reduced the number of candidates that were eventually sequenced to confirm the yeast ERG3 knock out.

All plasmids constructed were sequenced and verified with the sequences listed in their respected database. After transformation of the plasmid into each yeast strain, a diagnostic PCR of the respected gene confirmed the transformation (Data not shown). Yeast strains are listed in Table 2.

Protein Expression and Sterol Profile Analysis

Evaluation of the PcERG3 gene started with using the 300bp of the promoter region of Sc69 ERG3 to get expression of the genes in yeast. ScN2 (*P. capsici*) and ScN3 (*S. cerevisiae*) were not tagged and protein expression was low when directed by the Sc69 ERG3 promoter.

The protein was not visible by Western blot analysis. A diagnostic PCR showed that the genes were in the appropriate yeast strain, but the level of protein production was not determined (Figure 5). These strains were tested for ERG3 complementation through a phenotypic assay with cycloheximide. ScN3 and ScN7 were able to grow on the 20ppm cycloheximide, but ScN1 and ScN2 did not grow. However, when the sterols collected from these yeast strains were analyzed by LC/MS for the presence of ergosterol there are peaks found in both strains that match the relative retention time (RRT) and mass for ergosterol (Figure 6). The ergosterol peak in ScN2 is seen at RRT of 4.62 minutes while there is no peak seen at this RRT in ScN1. The amount of total sterols collected from each strain was not obtained, but as seen in Figure 6, ScN2 has very weak production of ergosterol when compared to ScN7. ScN3 produced ergosterol close to the levels found in ScN7.

A 1xFLAG-tag was added to the 3'-end of both the *P. capsici* and *S. cerevisiae* ERG3 gene to demonstrate that the protein was being expressed in ScN2 and ScN3. The FLAG-tag containing ERG3 strains were called ScN9 and ScN10, respectively. The production of protein in ScN9 was verified through Western blot analysis. A band was seen at the expected molecular weight (~34kD) for ScN9 but did not appear for ScN10 (Data not shown). The sterol profile for ScN9 showed a peak at a RRT of 4.68 minutes (Figure 7). A peak at this RRT matches the peaks seen in the samples of the ergosterol standard, the wild-type yeast profile and the Sc69 ERG3 complemented strain (ScN10). As with ScN2 and ScN3, the total sterols were not measured, but it is shown that ScN9 has weak ergosterol production as defined by LC-MS. This ergosterol production for both ScN9 and ScN10 was consistent with the levels seen in ScN2 and ScN3 respectively (Figure 7).

The Sc69 ERG3 promoter was replaced by a Gal1 promoter to increase the level of protein production; subsequently, ergosterol production in FL-PcERG3 complemented the yeast *erg3* null mutant [8]. ScN5 is the FL-PcERG3 complement while ScN6 is the Sc69 ERG3 complement. Only induced ScN5 showed protein expression at the expected molecular weight. Western blot analysis (Figure 8) showed that expression levels in ScN5 remained constant and did not increase with time (OD₆₀₀ 1.5 to 5.8). However, it was required to have a fresh transformation for ScN5 to show expression. The *FL-PcERG3* was eventually repressed and did not show expression in ScN5 (Data not shown). ScN6 did not express Sc69 ERG3 as determined by Western blot analysis. Sterol analysis from the same samples from the Western blot for ScN5 demonstrated a slow but steady increase of ergosterol production (Figure 9). This was inferred by comparing the difference over time of the ratio between the ergosterol peak and the major peak seen in a chromatograph at $m/z = 381.5$. This peak at $m/z = 381.5$ at a RRT of 4.93 minutes is the ergosterol pathway product found in *erg3* yeast mutants ((22E)-ergosta-7,22-dien-3Beta-ol). The ratio of ergosterol/((22E)-ergosta-7,22-dien-3Beta-ol) from the two sets of samples shows a trend of increasing amounts of ergosterol with a decrease in (22E)-ergosta-7,22-dien-3Beta-ol (Figure 10). The ergosterol production in ScN5 was still significantly less than production in ScN6.

ScN5 codon usage in FL-PcErg3 was analyzed after determining that protein expression impairs ergosterol production (Figure 11). Since the codon usage for FL-PcERG3 differs significantly from the codon usage preferred by yeast, a yeast codon-optimized version, designed and synthesized by GenScript, was obtained. pDN6 was created and transformed into Sc69 to make ScN8 [9]. The Western blot of ScN8 at an OD₆₀₀ 2.4 shows better expression of the putative *P. capsici* C5-desaturase (DES-5) than any time point from ScN5 (Figure 12). ScN8

continued to show weak production of ergosterol when compared to ScN6 but was better than ScN5 (Figure 12). The ratio of (22E)-ergosta-7,22-dien-3Beta-ol/ergosterol was measured from four separate samples of ScN8 that were taken at $OD_{600} \geq 2.9$ and averaged. These results were compared to the previous samples taken from ScN5 which were also over $OD_{600} 2.9$. ScN5 has a ratio of 81.3:1 ((22E)-ergosta-7,22-dien-3Beta-ol/ergosterol) with a standard deviation of 1.9 while ScN8 has a ratio of 24.5:1 ((22E)-ergosta-7,22-dien-3Beta-ol/ergosterol) with a standard deviation of 3.5 (Table 3).

When PcERG3 is expressed in ScN9, ScN5 or ScN8, there is production of the FLAG-tagged protein at the predicted size. These same samples also contained ergosterol as determined by LC/MS analysis. Ergosterol production was present when the FLAG-tag was located at the 3' or 5' end of the FL-PcERG3 gene and is also seen when using either the Sc69 ERG3 promoter and the Gal1 promoter. The sterol profiles show the small amount of ergosterol that is produced through the assistance of the *P. capsici* DES-5 and that when protein level is increased there is also an increase in ergosterol production. These results point to the conclusion that there is an ERG3 gene in *P. capsici* that can complement the ergosterol pathway in yeast.

Table 3.1. ClustalW values for a comparison of ERG3 genes. *N. tabacum*, *A. thaliana*, *S. pombe*, *H. sapiens*, *C. reinhardtii* have all been proven functional when expressed in *erg3* mutant strains of *S. cerevisiae*.

	<i>Phytophthora capsici</i>	<i>Phytophthora sojae</i>	<i>Sacharomyces cerevisiae</i>	<i>Nicotiana tabacum</i>	<i>Arabidopsis thaliana</i>	<i>Schizosaccharomyces pombe</i>	<i>Homo sapiens</i>	<i>Chlamydomonas reinhardtii</i>
<i>Phytophthora capsici</i>		85	50	22	27	52	53	22
<i>Phytophthora sojae</i>	85		50	23	25	53	51	24
<i>Sacharomyces cerevisiae</i>	50	50		22	24	51	43	21
<i>Nicotiana tabacum</i>	22	23	22		75	20	23	49
<i>Arabidopsis thaliana</i>	27	25	24	75		22	27	46
<i>Schizosaccharomyces pombe</i>	52	53	51	20	22		43	22
<i>Homo sapiens</i>	53	51	43	23	27	43		19
<i>Chlamydomonas reinhardtii</i>	22	24	21	49	46	22	19	

Table 3.2. Plasmids and yeast strains. This is description of the plasmids and yeast strains used in this thesis project. A. This is a table of the plasmids with their functional aspects listed. B. This is a table of the yeast strains with a description of plasmids associated with each.

A

Plasmid	Plasmid description
pRS425	2micron origin, LEU2
pJS564	2micron origin, TRP2, Gal1-3xFLAG-tag, cyc
pJS495	pRS425 + FL-PcERG3
pDN1	pRS425 with Sc69 ERG3 Promoter + FL-PcERG3
pDN2	pRS425 with Sc69 ERG3 Promoter + Sc69 ERG3
pDN3	pRS425 with Sc69 ERG3 Promoter + P. capsici ERG3 + 1xFLAG
pDN4	pRS425 with Sc69 ERG3 Promoter + Sc69 ERG3 + 1xFLAG
pDN5	pJS564 with Gal1 promoter + FL-PcERG3 + 1xFLAG
pDN6	pJS564 with Gal1 promoter + Sc69 ERG3 + 1xFLAG
pDN7	pJS564 with Gal1 promoter + 3xFLAG + Yeast Opt. FL-PcERG3

B

Strain	Yeast description
ScN1	ScN4 (KO) + pRS425
ScN2	ScN4 (KO) + pDN1 (Pc ERG3)
ScN3	ScN4 (KO) + pDN2 (Sc69 ERG3)
ScN4	Sc69 erg3 (KO with KanMX gene)
ScN5	ScN4 (KO) + pDN5 (Pc ERG3)
ScN6	ScN4 (KO) + pDN6 (Sc69 ERG3)
ScN7	Sc69 (WT) with pRS425
ScN8	ScN4 (KO) + pDN7 (Yeast codon opt Pc ERG3)
ScN9	ScN4 (KO) + pDN3 (Pc ERG3)
ScN10	ScN4 (KO) + pDN4 (Sc69 ERG3)

Table 3.3. Area under the curve from ScN5 and ScN8 samples. These samples show the raw counts for the samples taken from induced ScN5 and ScN8 at their indicted OD₆₀₀. This is followed by the ratio of two sterols. The average ratio value for ScN5 is 83.1 with a standard deviation of 1.9 while the average rate value for ScN8 is 24.8 with a standard deviation of 3.5.

	OD ₆₀₀	Ergosterol	(22E)-ergosta-7,22-dien-3Beta-ol	((22E)-ergosta-7,22-dien-3Beta-ol/ergosterol
ScN5	2.9	96186	7559562	78.59316325
	3.8	96869	8009295	82.68171448
	5.8	75884	6274824	82.68968425
ScN8	3.1	159241	4120785	25.87766342
	3	209978	4235230	20.16987494
	3	98201	2797195	28.48438407

Figure 3.1. Protein alignment with various ERG3 proteins. This protein alignment of the ERG3 protein sequences shows the conserved histidine regions seen in all ERG3 proteins (yellow highlight). It also highlights in grey single amino acid changes that have been shown to cause significantly reduced activity or complete loss of activity of the *Arabidopsis thaliana* ERG3. The TMHMM predicted transmembrane helices are in blue text while the UniProt predicted helices is in green text for *P. capsici* and *S. cerevisiae*, the two subjects of this study.

```

P. sojae -----
P. capsici -----MRLWNTSAPSRCMTIRGKDGA 21
H. sapiens -----
S. cerevisiae -----
S. pombe -----
N. tabacum -----
A. thaliana -----
C. reinhardtii MSTSLKMSATLARSLAVVAVFGAVLVSSHLLAGSAE VVHTKFPNVVIAGHSSSLLSGVRA 60

P. sojae -----MDLILEYADYYALD-AVYPADMP----- 22
P. capsici ASFTWQFPPVAKFAMDLILEYADYYALD-ALYPAYLP----- 57
H. sapiens -----MDLVLRVADYYFFTPYVYPATWP----- 23
S. cerevisiae -----MDLVLEVADHYVLD-DLYAKVLPASLAANI PVKWQKLLGLNSGFNS 45
S. pombe -----MDYLLNYADQYALD-SIYNAYYP----- 22
N. tabacum -----MEDYLKQFVEETS FYNRLVLTGTFMPES----- 27
A. thaliana -----MAATMADYNDQIVNETS FYNRMVLSHLLFPVN----- 31
C. reinhardtii WFASFATQADMPVAERLRLMREENEWKNNLVM-WLFPDS----- 98
                :           :           *

P. sojae -----RDSISRQIASITGITLVGGY 42
P. capsici -----RDDIYRQIASITGLTLVGGY 77
H. sapiens -----EDDIFRQAISLLIVTNVGAY 43
S. cerevisiae STILQETLNSKNAVKECRRFYGQVPLFDMSTTSFASLLPRSSILREFLSLWVIVITIFGL 105
S. pombe -----LARDNIVRQISISLFFLTWFGGM 44
N. tabacum -----WWGPLPHMLQGWLRNYIGGV 47
A. thaliana -----LWEPLPHFLQTWLRNYLAGN 51
C. reinhardtii -----VRDQIPHFWQTWLRCWILCA 118
                .           .           .

P. sojae LLYLIG----AGFAYQWFQDKELMKHPKFLKDQVKSEIVASLKSIPVMVMLTLPWFLADV 98
P. capsici LLYFIG----AGFAYEFLFDKELMNHKPKFRKNQVRSEILFSMKSIPGMVLLALPWFLADV 133
H. sapiens ILYFFC----ATLSYFVFDHALMKHPQFLKNQVRREIKFTVQALPWI SILTVALFLEI 99
S. cerevisiae LLYLFT----ASLSYFVFDKSI FNHPRYLKNQMAMEIKLAVSAIPWMSMLTVPWFVME 161
S. pombe FLYLTF----ASLSYQFVFDKSLMDHPKFLKNQVFMVLTALQNLPGMALLTVPWFLAEL 100
N. tabacum LLYFISGFLWCFYIYHLKRNVIYIPKDAIPSNKAMLLQISVAMKAMPWYCALPSLSEYMIE 107
A. thaliana ILYFISGFLWCFYIYLLKLNVIYVPEKSIPTRKAMLLQIYVAMKAMPWYTLPAVSEYMIE 111
C. reinhardtii AVYFGVGLWCYTYFCFGDKWFAPGTIPAWKDVAEQMRVSNIAMPLYSMLPALTEMAAE 178
                :* : . * : . . . : : : : * * .

P. sojae RGYSKTYTEFGKYGYGFEAMAV--VWFVFFSDMLIYWFHRWLH-HPLIYAPLHKPHHKWI 155
P. capsici RGFSKTYTDFGKFGYGFALS SV--VAFICFSDMLIYWFHRWLH-HPLIYAPLHKPHHKWI 190
H. sapiens RGYSKLHDDLGEFPYGLFELVVSIIISFLFFTDMMFYWIHRGLH-HRLVYKRLHKPHHKWI 158
S. cerevisiae NGHSKLYMKIDYENHGVKRLIIEYFTFIFFTDGCVYLAHRWLH-WPRVYRALHKPHHKWL 220
S. pombe HGYSYLYDNIS--DYGKLYFLCPLFVMSDFGIYWAHRFLH-HRYVYPRLHKLHKHWI 157
N. tabacum NGWTKCFARIS--DVGWLSYVIAIYLVIVEFGIYWMHMLHDIKPLYKYLHATHHIYN 165
A. thaliana HGWTKCYSTLD--HFNWFLCFLYIALYLVLFVFMFYVWVKELHDIKFLYKHLHATHHMYN 169
C. reinhardtii KGWTRAYPRVE--NVGLPAYVLYFFLYMTSVEFGVYWMHRILH-WGPLYKYLHWDHKKYN 235
                .* : . . . : : : * * ** : * ** * :

P. sojae ---ICSPFASHAFHPVDGYIQSLPYHFYIMLFPPIHRGLFLALFAAVNFWTISIHGDGLYLS 212
P. capsici ---VCSPPFASHAFHPVDGYIQSLPYHFYIMLFPPIHRGLFLALFVAVNFWTISIHGDGYLS 247
H. sapiens ---IPTPFASHAFHPIDGFLQSLPYHIYPFIFPLHKVVYLSLYILVNIWTISIHGDGFRV 215
S. cerevisiae ---VCTPFASHAFHPVDGFLQSLISYHIYPLILPLHKVSYLILFTFVNFVWTVMIHGDQYLS 277
S. pombe ---ICTPYASHAFKSDGFLPYHLFPFFPLHKLTYLALFTFVNFWSIMIHGDGKYIS 214
N. tabacum KQNTLSPFAGLAFHPLDGIQAVPHVVALLLVPMHFSTHIALIFLEALWTANIHDICIHGK 225
A. thaliana KQNTLSPFAGLAFHPLDGIQAIHPHIALFVPIIHLITHLSLLFLEGIWTASIHDCIHGN 229

```

```

C. reinhardtii    KEHTLSFFAGLAFHPLDGILQAVPYAWTLLYCPMHFLTHELLLAFATGVWTTNIHDCLHGH 295
                  :*:. :*:. ** :*:.: : *:* . * .*: *** .

P. sojae         ---HNKVVNGALHHSVHHELFVYNYGQYFTLWDRLCGSY-----PKKKL----- 253
P. capsici       ---HNKIVNGARHHSVHHEQFVYNYGQYFTFWD RMFGSYREPPTTGYSIPKET----- 297
H. sapiens       PQILQPFINGSAHHTDHHMFFDYNYGQYFTLWDRI GGSFKNPSSFEGKGPLSYVK----- 270
S. cerevisiae    ---NNPAVNGTACHTVHHLYFNYNYGQFTTLWDR LGGSYRRPDDSLFDPKLRDAKETWDA 334
S. pombe         ---NNPIINGAAHNGHHIYFNYNYGQFTTLF'DRLGNSFRAPDEAWFDKDLRQNE DVLRV 271
N. tabacum       ---VFPVMGAGYHTIHHRTYRHNYGHYTIWMDWMFGTLRDP-VEEDAKKM----- 271
A. thaliana      ---IWPIMGAGYHTIHHTTYKHNYGHYTIWMDWMFGSLMVPLAEKDSFKEKEK----- 279
C. reinhardtii   ---VWPIMGAGYHTIHHTTYKHNYGHYFIFMDYLMGTLTTP-EQYDADKAARATKAVKA 350
                  : * : * . ** : :***: : * : .:

P. sojae         -----
P. capsici       -----
H. sapiens       EMTEGKRSSHSGNGCKNEKLFNGEFTKTE-- 299
S. cerevisiae    QVKEVEHFIKEVEGDDNDRIYENDPNTKKN 365
S. pombe         ELMEYEAIRNEVEGDD-DREYIAN-SAKKNH 300
N. tabacum       -----
A. thaliana      -----
C. reinhardtii   Q----- 351

```

Figure 3.2. TMHMM analysis of the *S. cerevisiae* and *P. capsici* ERG3 enzymes. A. Three transmembrane helices are predicted for the *S. cerevisiae* ERG3, but there is a fourth potential helix shown that does not meet the probability cut-off. B. *P. capsici* ERG3 has 4 helices predicted.

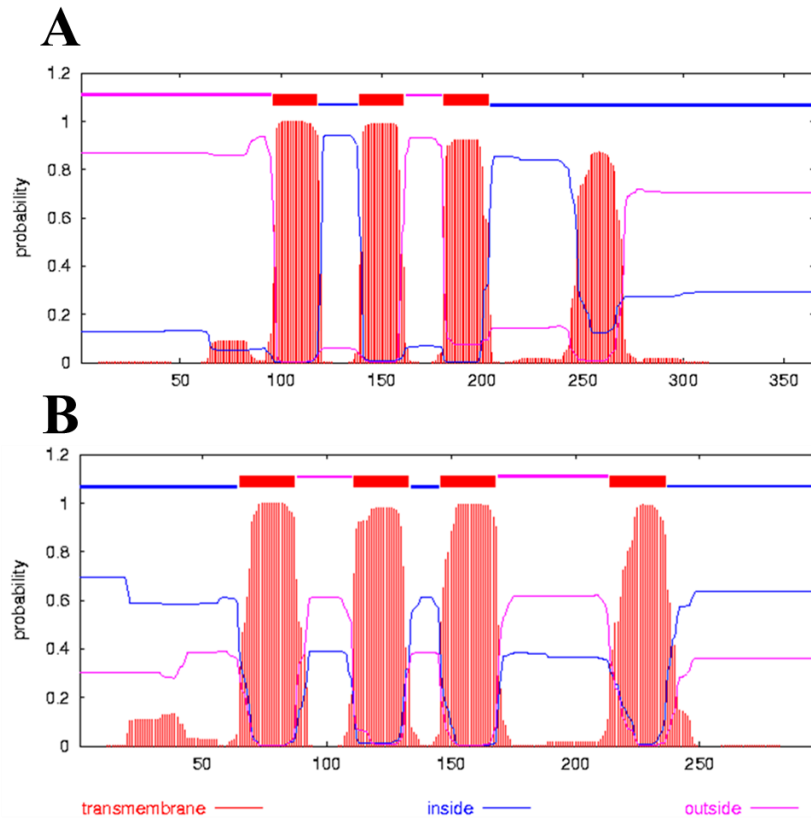


Figure 3.3. Alignment of *Arabidopsis thaliana* DWF5 with potential C7 reductase found in *P. capsici*. There is 94% coverage with 54% identity and 66% similarity. The ERG4/ERG24 family alignment in *P. capsici* is found from amino acid 61 to 432 and the transmembrane helices predicted by TMHMM are in blue text.

```

P capsici: 22  LFLMTVTPVAATLITHVFVQHGGSSINA----FLASG-KSLIEIWPTPWDPTAWKLIQVFM 76
                L L+  P   L+ + V  GS+   F  +G + LI IWP P   AWK+I  +
A thaliana: 16  LSLLAFCPPFVILLWYTMVHQDGSVTQTFGFFWENGVQGLINIWRP-TLIAWKIIFCYG 74

P capsici: 77  VAQLFLMRAVPGKPSYGPITPGGNVPVYKANGFQCFALSLALYLAGAFVFELYPGGIVYD 136
                +  L   +PGK  GPI+P GN PVYKANG  + ++LA YL G + F ++  IVYD
A thaliana: 75  AFEAILQLLLPGKRVEGPISPAGNRPVYKANGLAAYFVTLATYL-GLWWFGIFNPAIVYD 133

P capsici: 137 YMPEIISALNVFSYIILCLFLYIKGAFLOWSSSDCGTSGNPVDFYWGTELYPRV-LGWDI 195
                ++ EI SAL   S+I C+ LYIKG      SSSD G+ GN + DFYWG ELYPR+  +DI
A thaliana: 134 HLGEIIFSALIFGSFIFCVLLYIKGHVA-PSSSDSGSCGNLIIDFYWGMELYPRIGKSFDI 192

P capsici: 196 KQFTNCRCLMFWAVGVLSYACKQREMYGYLSDSMAVSVLQLVYVAKFFWWEAGYMCSI 255
                K FTNCR G+M WAV  ++Y  KQ E+ G +SDSM V+  L LVYV KFFWWEAGY  ++
A thaliana: 193 KVFTNCRFGMMSWAVLAVTYCIKQYEINGKVSDSMLVNTILMLVYVTKFFWWEAGYWNTM 252

P capsici: 256 DIMHDRAGYYLCWGCLVWVPSVYTSYAMYLQNPVHGLTPLALAIKITGVLVVGINYDAD 315
                DI HDRAG+Y+CWGCLVWVPSVYTSY MYLV +P+ LGT LA+ I + G+L + INYD D
A thaliana: 253 DIAHDRAGFYICWGCLVWVPSVYTSYSPGMYLVNHPVELGTQLAIYILVAGILCIYINYDCD 312

P capsici: 316 RQRQGFRAANGKTNVWGRPAEFIVARYETEKGEKKQSLLLTSGWWGLARHFHYVPEVLAA 375
                RQRQ FR  NGK  VWGR   IVA Y T  GE K SLLLTSGWWGLARHFHYVPE+L+A
A thaliana: 313 RQRQEFRRRTNGKCLVWGRAPSKIVASYTTTSGETKTSLLLTSGWWGLARHFHYVPEILSA 372

P capsici: 376 VSWTIPALGSSPAPYFYSIFLAILLTDRAYRDDARCAHKYRQDWKKYCERVPSLILPKI 434
                WT+PAL  +   YFY IFL +LL DRA RDD RC  KY + WK YCE+V   I+P I
A thaliana: 373 FFWTVPALFDNFLAYFYVIFLTLLLFDRAKRDDDRCSRKYGKYWKLYCEKVYRIIPGI 431

```

Figure 3.4. Diagnostic PCR results for ScN4. (A) This shows a diagram of the KanMX gene with the flanking Sc69 ERG3 promoter and terminator regions that was used for knocking out the ERG3 gene. It includes the primer numbers used and the location of each primer in reference to the start ATG. (B) The gel shows the PCR results for the primer sets listed in yellow when run with either gSc69(Wild-type yeast) or gScN4 (yeast *erg3* null mutant). Lanes 1 and 2 show the PCR reaction of 1573 and 2833 with gSc69 (1890 bp) and gScN4 (2190 bp), respectively. Lanes 3 and 4 show the PCR reaction of 1573 and 2832 with gSc69 (0 bp) and gScN4 (1285 bp), respectively. Lanes 5 and 6 show the PCR reaction of 2831 and 2833 with gSc69 (0 bp) and gScN4 (1382 bp), respectively.

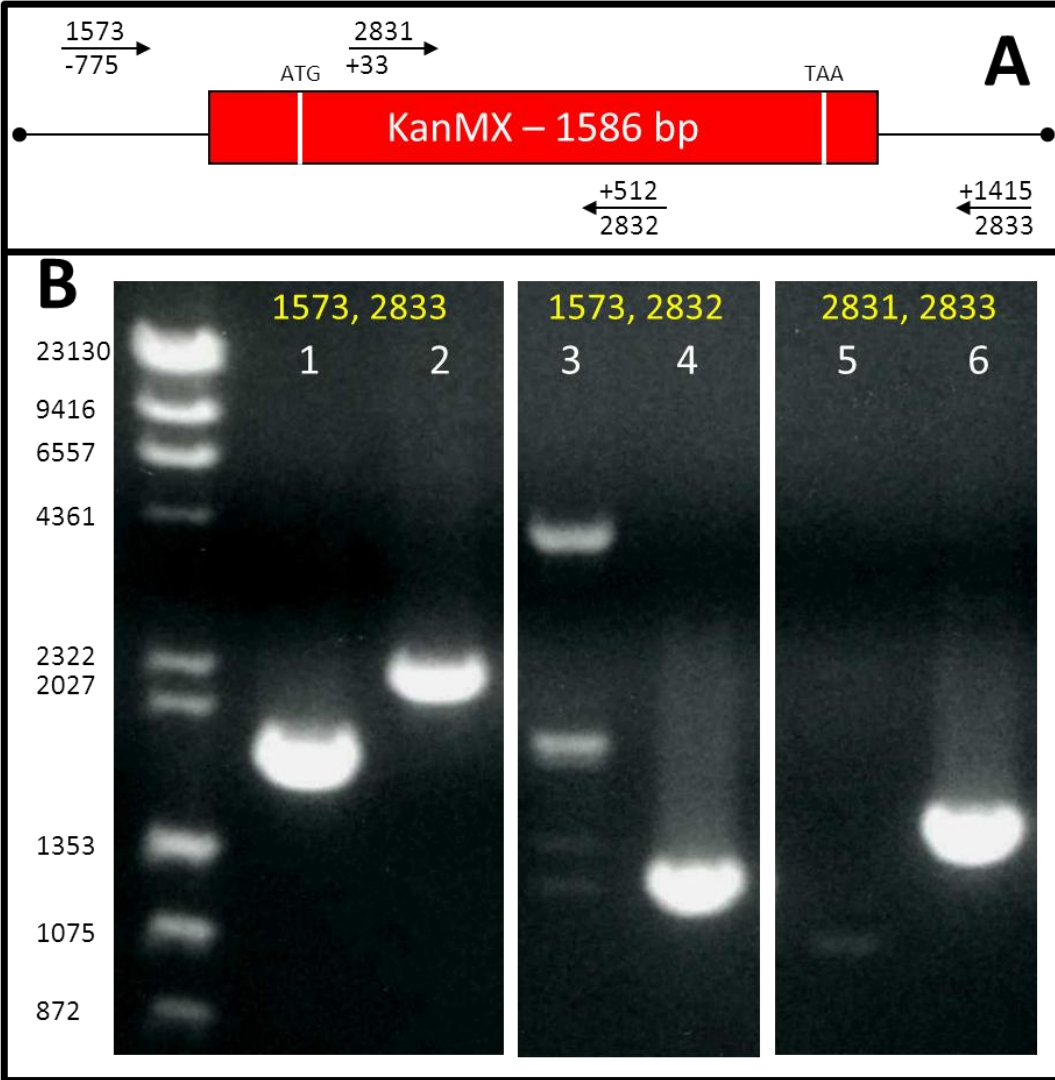


Figure 3.5. Diagnostic PCR for ScN2 and ScN3. A. Diagram of the genes found in ScN3 (Sc69 ERG3) and ScN2 (PcERG3). Both genes have 300bp of the Sc69 promoter region and Sc69 ERG3 also has 300bp of Sc69 ERG3 terminal region. The primers list their title and their distance in relationship to the start ATG. B. Lanes 1-5 and lane 7 shows the reaction with primers 1573, 1602 with gSc69 (1662bp), gScN4 (2098bp), pDN2 (1662bp), pDN4 (0bp), gScN3 (2098 and 1662 bp) and gScN2 (2098bp). Lane 6 shows the reaction of 1618 and 1602 with gScN6 (850bp). Lane 8 is a reaction with primers 1573 and 1840 using gScN2 (1150).

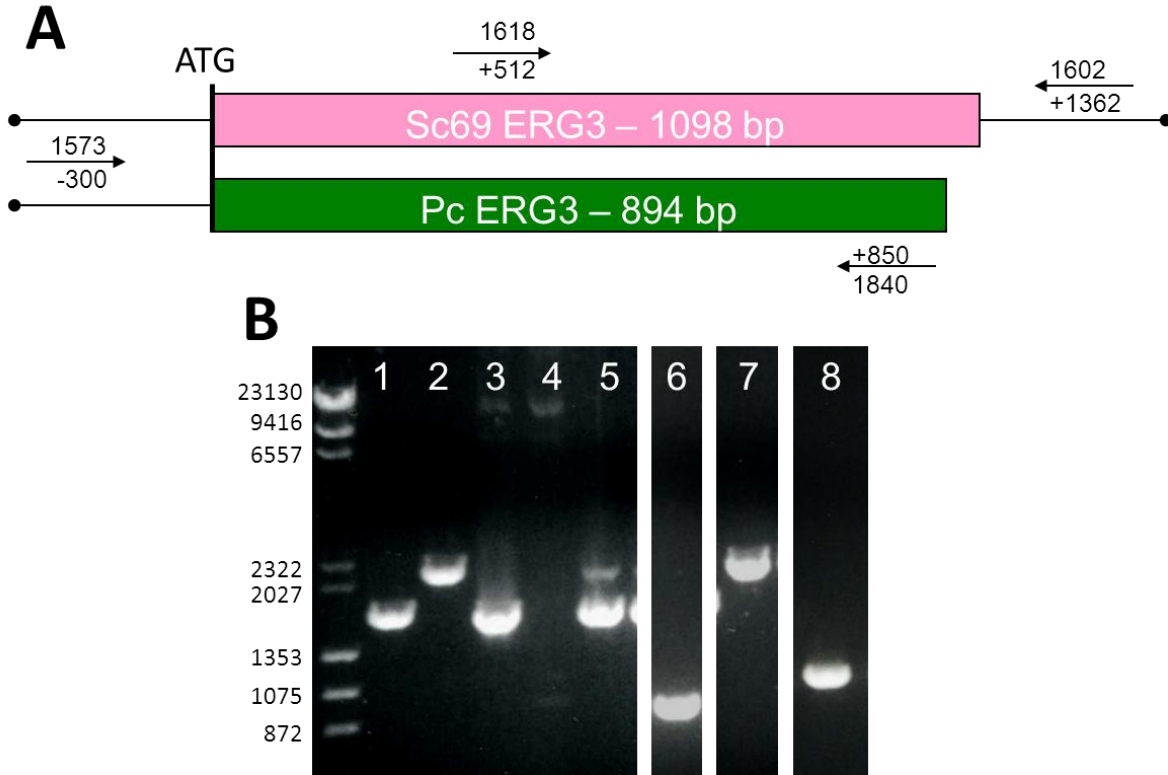


Figure 3.6. HPLC/MS data for ergosterol standard, ScN7, ScN1, and ScN2. These chromatographs show the sterol profile from a 20ppm sample of ergosterol (A), a sample of ScN7 (B), a sample from ScN1 (C), and a sample from ScN5 (D). Each chromatograph is taken from a mass of 379.5 which is the molecular weight of ergosterol.

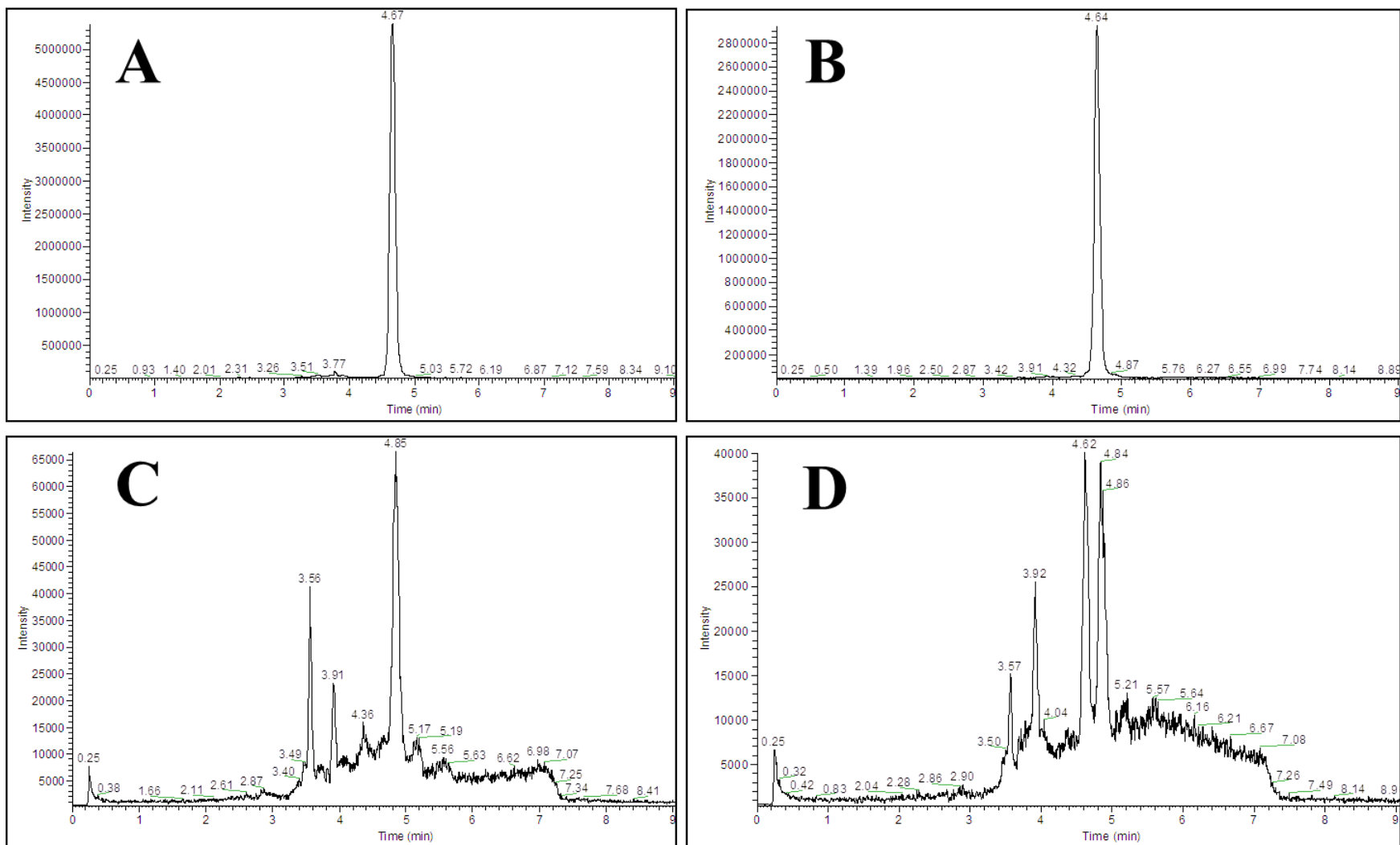


Figure 3.7. HPLC/MS data from samples of ScN1, ScN7, ScN9 and ScN10. A. This depicts the difference seen when both masses 379.5 (ergosterol) and 381.5 ((22E)-ergosta-7,22-dien-3 β -ol) are selected for a chromatograph from a sterol sample of ScN7. B. This is the same chromatograph settings as A, but the sample is from ScN10. C. A chromatograph from ScN9 at a mass of 381.5. D. Shows a sample from ScN1 when only a mass of 379.5 is selected. E. A chromatograph from ScN9 when 379.5 is selected.

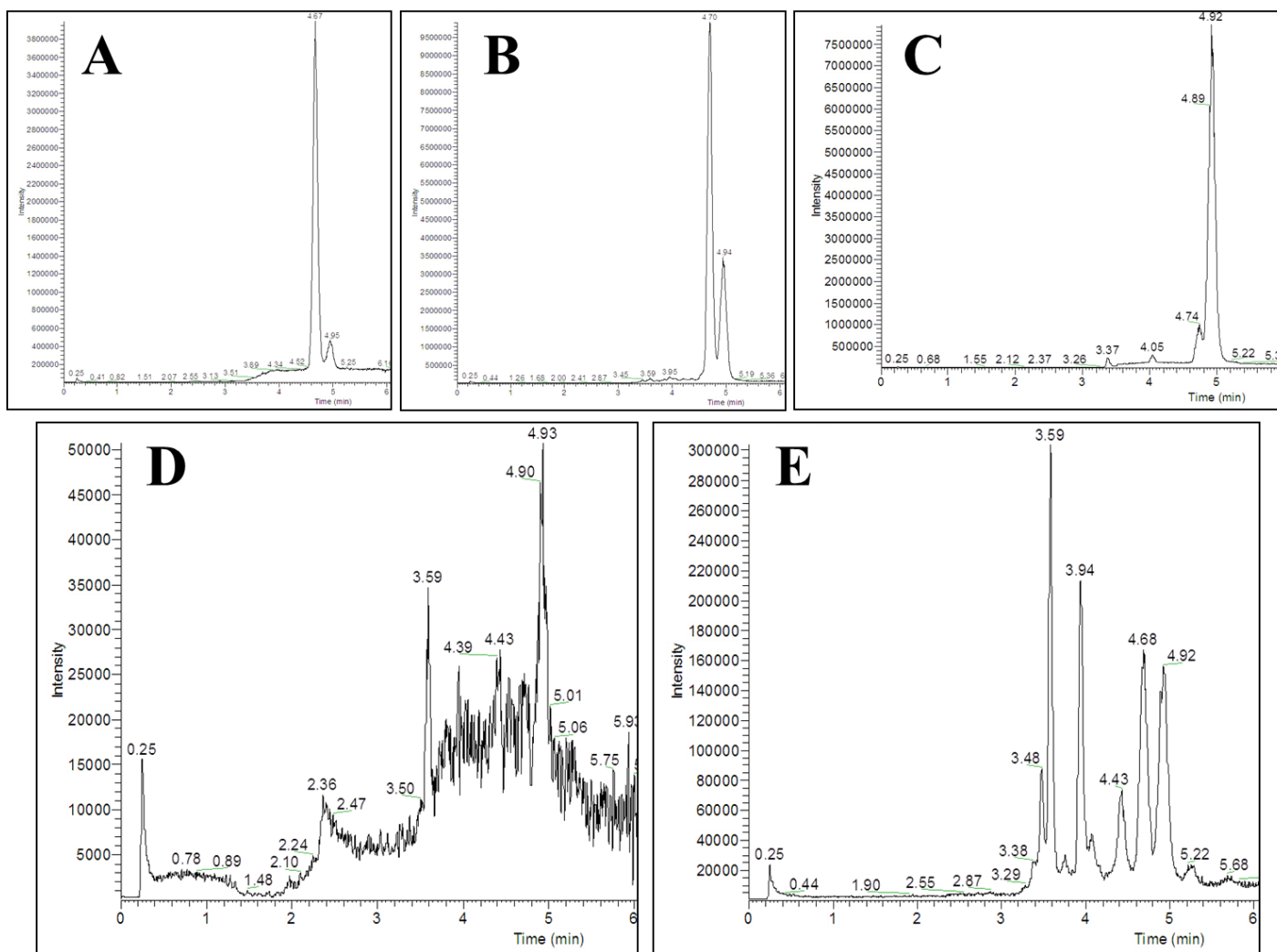


Figure 3.8. Western Blot analysis of ScN5 and ScN8. Each sample had 20ug of protein loaded per lane and both films were exposed to their respective blot for 4 minutes. The expected size of the putative *P. capsici* DES-5 is ~34kD. A. A film with ScN1 (OD₆₀₀ = 6.9) in lane 1, induced ScN5 (OD₆₀₀ = 1.5, 2.5, 2.9, 3.8 and 5.8) in lanes 2-6, ScN5 (OD₆₀₀ = 1.4, 3 and 5) in lanes 7-9. B. A film showing induced ScN8 (OD₆₀₀ = 2.4), ScN6 (OD₆₀₀ = 5.6), ScN1 (OD₆₀₀ = 6.5) and ScN7 (OD₆₀₀ = 6.9).

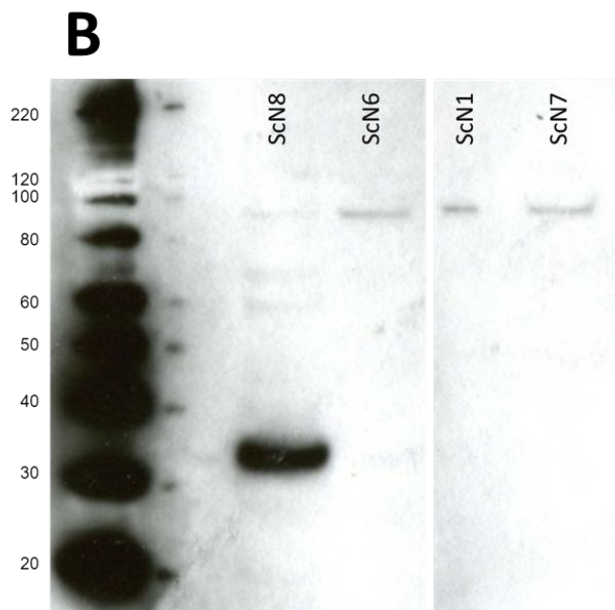
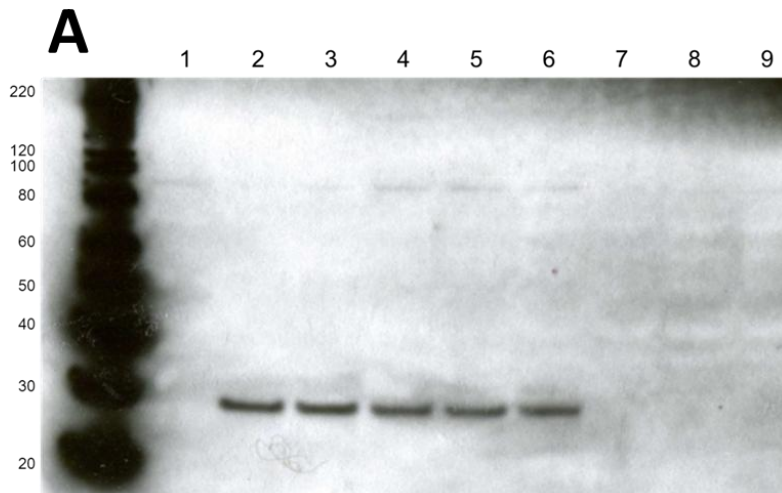


Figure 3.9. Chromatographs showing non-induced compared to induced ScN5 at different OD₆₀₀ values. Each section displays the non-induced chromatograph followed by the induced chromatograph for mass 379.5. The OD₆₀₀ values are 1.5, 3.0, 4.2, and 5.2 for A, B, C and D respectively.

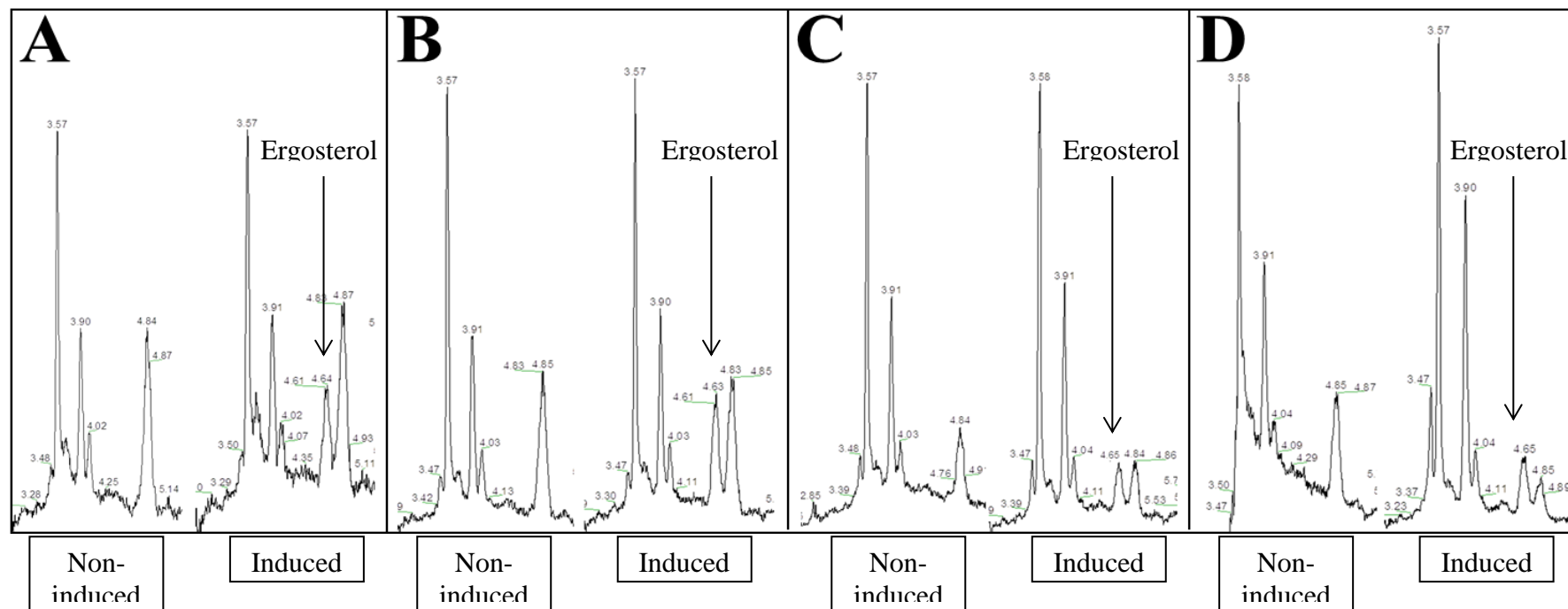


Figure 3.10. Graph of the ratio of area under the curve of ergosterol divided by the area under the curve of (22E)-ergosta-7,22-dien-3Beta-ol plotted over time. This graph shows that over time, there is an increase of ergosterol while there is a decrease in (22E)-ergosta-7,22-dien-3Beta-ol. These samples were taken from two separate growths of induced ScN5.

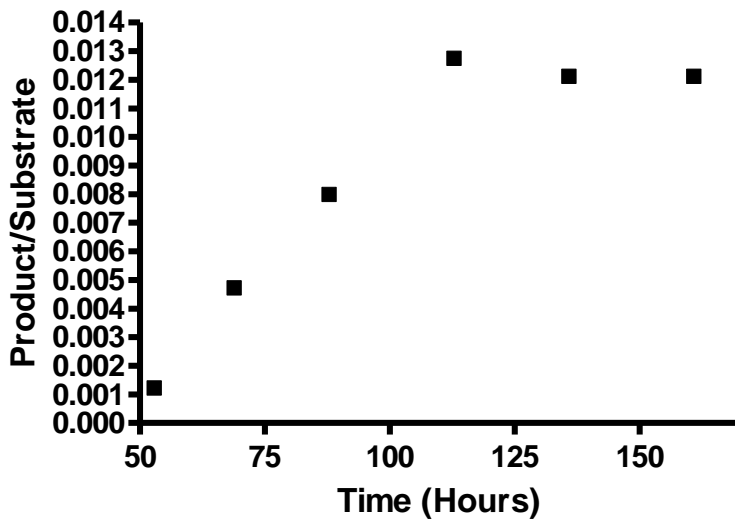


Figure 3.11. Rare codon graphic. Graphic representation of codon preference of either PcERG3 (A) or the yeast optimized PcERG3 (B). These graphics were produced by the online rare codon calculator from the Clark Lab at the University of Notre Dame [10]. This program uses a sliding 18 codon window to analyze sequences (window 1 = codons 1 to 18, window 2 = codons 2 to 19, etc.). The x-axis shows the codon window analyzed while the y-axis shows the percent of codons that are rare (%Min = negative/red) or common (%Max = positive/blue) over the 18 codon window analyzed.

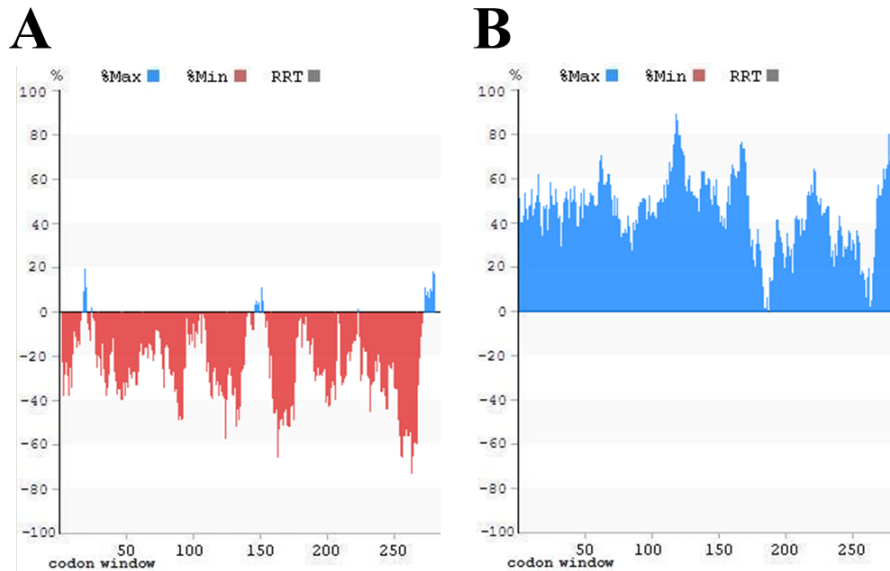
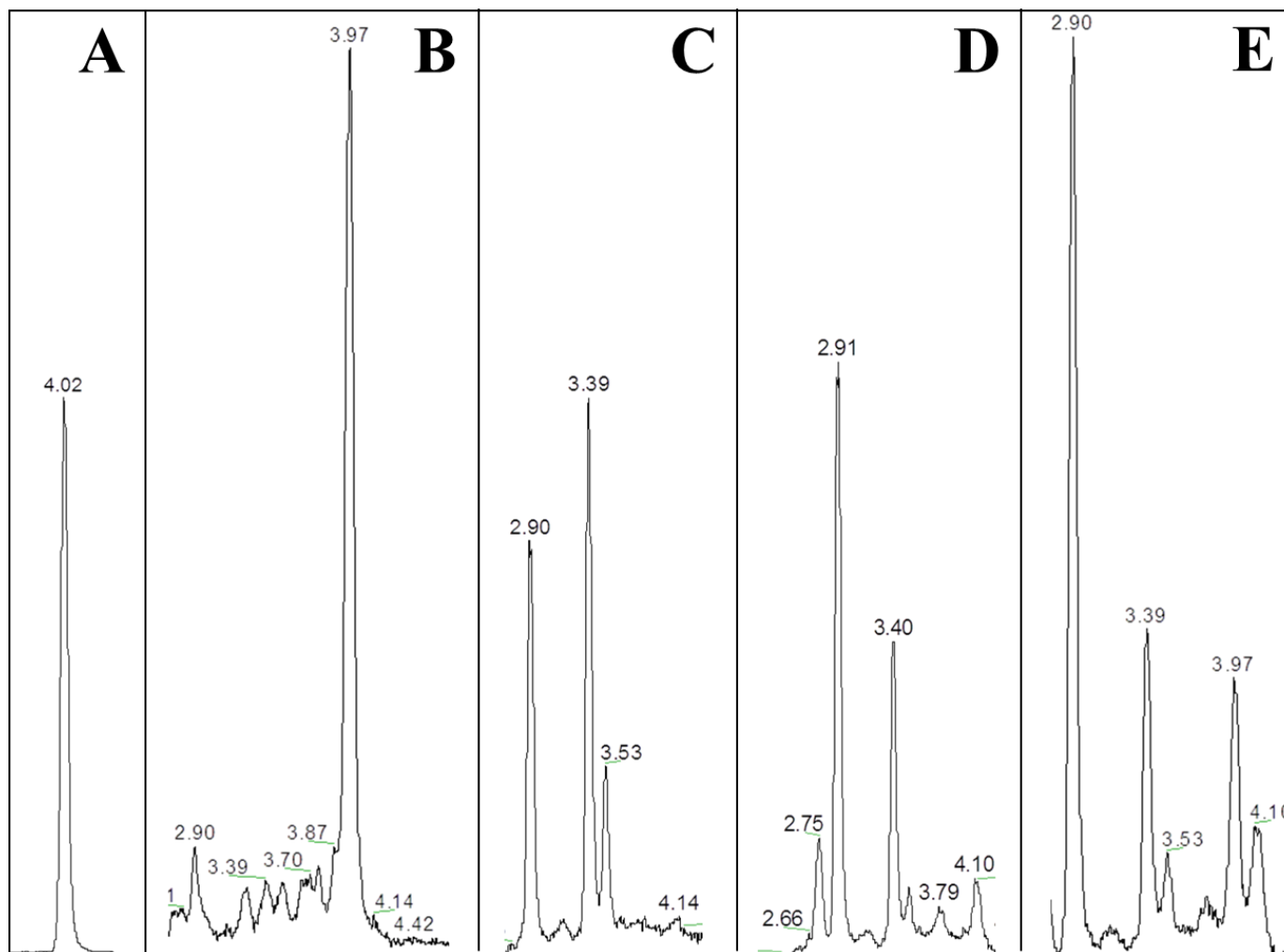


Figure 3.12. HPLC/MS of induced and non-induced ScN8. Sterol profiles isolated from mass 379.5. A. Ergosterol standard was assayed at a rate of 200ppm to provide an accurate RRT and mass for the other assays. B. Shows a chromatograph from induced ScN6. C. A chromatograph from non-induced ScN8. D. ScN1 confirms the profile for the non-induced ScN8. E. Induced ScN8 shows a mild complementation profile.



REFERENCES

1. Altschul, S. F., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.
2. Brumfield KM, et al. (2010). Functional characterization of the *Chlamydomonas reinhardtii* ERG3 ortholog, a gene involved in the biosynthesis of ergosterol. *PLoS One* 5(1):e8659
3. Sugawara, T., et al. (2001). Molecular cloning and structural analysis of human sterol C5 desaturase. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids.* 1533(3): 277-284.
4. Gachotte, D., et al. (2002). Isolation and characterization of an *Arabidopsis thaliana* cDNA encoding a Δ 7-sterol-C-5-desaturase by functional complementation of a defective yeast mutant. *The Plant Journal.* 9(3): 391-398.
5. Husselstein, T., et al. (1999). Δ 7-sterol-C5-desaturase: molecular characterization and functional expression of wild-type and mutant alleles. *Plant molecular biology.* 39(5): 891-906.
6. Iwaki, T., et al. (2008). Multiple functions of ergosterol in the fission yeast *Schizosaccharomyces pombe*. *Microbiology.* 154(3): 830-841.
7. Sonnhammer E. L. L., et al. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology.* 6(6912): 175-182.
8. Winzeler EA, et al. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 285: 901-906.
9. Flick, J. S., et al. (1990). Two systems of glucose repression of the GAL1 promoter in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 10: 4757-4769.
10. Clarke T.F., et al. (2001). Rare Codons Cluster *PLoS ONE* 3: e3412.

Chapter 4

DISCUSSION

Genomic and Protein Sequence

Results from this study suggest that *P. capsici* has a functional ERG3 that can produce ergosterol in a yeast null mutant. The first evidence for an *ERG3* gene in *P. capsici* lies in the genomic and annotated protein sequences that are available for this organism. The BLASTN analysis of the *P. capsici* ERG3 gene in the Saccharomyces Genome Database shows 60% identity to the *S. cerevisiae* ERG3 with 5% gaps. Protein expressed from this sequence has 46% (BLASTP) or a 50 (ClustalW) score which is comparable to or better than some of the other DES-5 that have been used to complement the ergosterol pathway in *erg3* mutants [1]. The best comparable DES-5 predicted through a ClustalW analysis is found in *S. pombe* (51%) while the DES-5 with the least identity is *C. reinhardtii* (21%) [2]. *P. capsici* DES-5 has very good identity when compared to other DES-5 that have been shown to complement ERG3 activity in yeast null mutants.

Several conserved regions in the ERG3 protein have a significant effect on enzyme activity. First are the conserved histidine regions that have been studied in *A. thaliana* and *Zea mays* and are found in all DES-5. There were two studies that pointed to these conserved histidines being important for conformational reasons and not for catalytic reasons. Although the function of the histidine regions is still not defined, the importance of these amino acids and the few other conserved amino acids found by Taton et al. (2000), are crucial for enzymatic activity

of DES-5 [3, 4, 5]. For *P. capsici* DES-5, each of these conserved amino acids is in alignment with the each of the DES-5 studied to date.

One of the other features predicted in the *P. capsici* DES-5 enzyme are four transmembrane helices. The function of these helices is not known, but they do provide some evidence that the *P. capsici* DES-5 is similar to *S. cerevisiae* DES-5. These predicted helices are good evidence that the *P. capsici* is membrane-bound like the *S. cerevisiae* DES-5. Also, when these two proteins are aligned using ClustalW, the first three helices align approximately at the same locations. There is evidence of a fourth helix in the *S. cerevisiae* ERG3 enzyme. TMHMM results are based on a hidden Markov model which uses an algorithm to evaluate the probability that each amino acid is in either helix, inside or outside [6]. The program then evaluates that data and produces a plot that shows the certainty of each helix. The fourth helix is shown to have a ~80% probability of being a helix region. The additional supporting data for this fourth helix is UniProts (<http://www.uniprot.org/uniprot/P32353>) prediction, which is a pooled from a variety of sources. So, assuming this fourth helix is real, it is also approximately at the same location as the fourth helix found in *P. capsici* DES-5.

Cytochrome b5 (CTB5) is a documented cofactor for this reaction [7]. It is part of the electron-transport chain and via NADH provides an electron necessary for the desaturase reaction. In *P. capsici* there is a sequence that has similarity to many other CTB5 genes. The BLASTP results of *P. capsici* CTB5 with *A. thaliana* and *S. cerevisiae* are 56% and 63% similarity respectively. The *P. capsici* CTB5 protein is predicted to have the appropriate N-terminal transmembrane helical that is seen in all other membrane bound CTB5 [7]. The interaction between the *S. cerevisiae* CTB5 may affect the functionality of the *P. capsici* DES-5 and is a possible contributing factor for the weak activity seen in our results.

C7 reductase (DWF5) is the other potential sterol pathway enzyme from *P. capsici*. It has 66% similarity in a BLASTP comparison with *A. thaliana* DWF5 and has similar TMHMM predicted transmembrane helices as the *A. thaliana* DWF5. The relationship of this enzyme with ERG3 in *P. capsici* is not well documented, but there have been studies in *Z. mays* [4] and *A. thaliana* [3] that give clues to their relationship. In higher plants, the sterol pathway involves conservation of Δ^7 -sterol to $\Delta^{5,7}$ -sterol to Δ^5 -sterol. The absence of a double bond at C7-C8 has been shown to inhibit C5-desaturase activity in *Z. mays*. There are hypotheses as to why the double bond at C7 and C8 is required in higher plants. Either the allelic C6 hydrogen activation is dependent on the C7-C8 double bond and/or there are conformational aspects of the enzyme that increase specificity. This suggests that *P. capsici* uses the typical sequence of sterol reactions found in all sterol synthase pathways, C5 desaturase to C7-reductase.

Protein Expression

The expression of ERG3 in ScN2 and ScN9 was very limited. ScN2 did not have a FLAG-tag and the protein expression was not substantial enough to be visualized for SDS-PAGE stained gel. The addition of the 1xFLAG-tag to the 3' end of *P. capsici* DES-5 in ScN9 allowed for detection of the expressed protein in yeast under the Sc69 ERG3 promoter by Western analysis. It has been documented in a paper by Higgins et al. (2003) that enzymes in the ergosterol pathway are repressed in the later stages of fermentation. In part of their study, they did a Northern blot of the ERG10 and ERG3 genes at 1 hour and 23 hours after starting an industrial fermentation [8]. At 1 hour, both genes showed expression but by the twenty-third hour, ERG10 was not being expressed and ERG3 had limited expression. So as yeast cultures

enter the lag phase of growth, the ergosterol pathway is repressed [8]. The Sc69 ERG3 promoter provided expression during sterol pathway and produced small quantities of *P. capsici* DES-5.

Based on Western blot data and HPLC/MS data, the Sc69 ERG3 promoter drove expression of both the Sc69 DES-5 and the *P. capsici* DES-5. Due to the low protein expression seen in ScN9 in the Western blot, the *P. capsici* ERG3 was placed under the strong inducible promoter Gal1 [9]. Gal1 is an inducible promoter so it can be used to compare protein expression and sterol profiles of a strain with or without sterols. Further, it is strong promoter so it should increase the level of protein found in the induced strain. When *P. capsici* DES-5 was induced there was constant production of the enzyme as determined by Western blot analysis.

Expression of the *P. capsici* DES-5 was limited under both the Sc69 ERG3 promoter and the Gal1 promoter. Increasing the production of *P. capsici* DES-5 was eventually accomplished by codon optimizing the gene for yeast. When the *P. capsici* FL-ERG3 gene is evaluated by the online Rare Codon Calculator from the Clark Lab at the University of Notre Dame, it is found to have 93% of the gene in the %Min window. This calculation is not an explicit predictor for translation rates, but it does provide evidence that there are rare codon clusters found in the *P. capsici* FL-ERG3 gene that may cause poor translation in yeast [10]. GenScript performed codon optimization for the *P. capsici* FL-ERG3 and a Western blot of protein from ScN8 showed increased expression of *P. capsici* DES-5 over that of ScN5.

Sterol Profile

The first objective in analyzing sterols through either GC/MS or HPLC/MS is to obtain a clean sterol sample. Originally, Bligh and Dyer created a lipid extraction for GC/MS [11]. This method involved adding a 1:2 solution (methanol:chloroform) to the collected cells. This would

lyse the cells and allow the addition of an internal standard. Another volume of chloroform is added and then the mixture is mixed thoroughly. A volume of pure water is added to wash the lipid sample. This is centrifuged for 5 minutes and the bottom organic phase is collected. This washing process can be done several times to clean the sample of any material which is water soluble. After the washing steps, the organic phase is evaporated under nitrogen gas and the lipids recovered can be stored at -80°C . Variations of this protocol have been used and provided steps that were used in this thesis. The protocol used in this thesis is an abridged form of what is described above but still provided a sample that separated the ergosterol peak at $m/z = 379.5$ and the (22E)-ergosta-7,22-dien-3Beta-ol peak at $m/z = 381.5$. This procedure extracted the lipids and sterols in a quick, reliable and usable form for this study.

After achieving a clean sample, the next step to consider is the column to use for the HPLC. The column used in this thesis was Atlantis dc18 IS column. This is a reverse-phase column which uses difunctional C18 ligands which can be used in aqueous conditions as well as in organic conditions. Separation of the ergosterol and (22E)-ergosta-7,22-dien-3Beta-ol was based on the hydrophobic interaction between the C18 matrix and the hydrophobic ring structure of the sterols. Due to the larger pore size, 5 μm instead of 3 μm , a higher flow rate can be applied without compressing the silica bed. This provided a shorter run time, enabling a higher number of samples that can be run per day. There are other potential columns that could be used for these experiments, but this column provided the adequate peak separation in a reasonable amount of time.

Another consideration for this assay is the organism to evaluate the complementation. Ideally, using *P. capsici* would be the best option but there are a couple of problems with using this organism. First is the lack of knowledge about the sterol pathway in any *Phytophthora spp.*

As already mentioned, there are feeding assays that have been conducted that implicate an abridged sterol pathway, but there are no definitive studies on these two potential genes (ERG3 and DWF5). The second reason not to use this organism is the difficulty faced when trying to make a *P. capsici* *erg3* null mutant. Currently there is no methodology for knocking a gene out of a diploid *Phytophthora spp.* Studies on *Phytophthora infestans* have shown gene silencing to be a possibility, but due to gradual re-activation of the silenced gene, this method would not be appropriate for this initial stage of study [12, 13, and 14]. So the most commonly used organism for the study of the sterol pathway is *S. cerevisiae*. The yeast system has been used for complementation of numerous genes in the ergosterol pathway. Arabidopsis sterol pathway genes have been used to complement the ERG2 (Δ^8 - Δ^7 isomerase), ERG3, ERG24 (Sterol C14 reductase) and ERG25 (sterol 4alpha-methyloxidase) in the respective yeast mutants [15, 16, 17]. The low similarity scores seen for each of these *A. thaliana* genes demonstrates the versatility of the ergosterol pathway in yeast. The ERG3 genes from *H. sapiens*, *C. reinhardtii*, *N. tabacum*, and *S. pombe*, have also complemented the *erg3* yeast mutants [18, 19, 20, and 21].

Using *S. cerevisiae* provides a user friendly system for evaluating *erg3* mutants. There are numerous phenotypic studies that can be conducted to establish if the ergosterol is being produced. The most frequently used phenotypic response is the yeast cells response to cycloheximide. This compound is very toxic to *erg3* yeast so if there is complementation the yeast cells should be able to grow in the presence of cycloheximide at low concentrations (~20ppm). The increased toxicity of the cycloheximide is believed to be a result of a porous cell membrane found in *erg3* yeast which allows cycloheximide in at a lower concentration [22]. This creates variability in the test because it is measuring how porous the membrane is and not the direct functionality of the ERG3 enzyme.

Another known phenotypic response is fluconazole resistance in *erg3* yeast [23]. This creates the scenario where *erg3* mutants can grow in the >256ppm fluconazole, while if there is complementation of the pathway, they will not be able to grow. Resistance in *erg3* mutants is because this mutant does not produce the final toxic compound that is found when the DES-5 is functional. There is an accumulation of the functional 14 α -methylfecosterol while if the DES-5 is functional, 14-methyl-3,6-diol is produced. In a study by Palusznski et al. (2008), they found that some of the fluconazole resistant mutants tested had leaky *erg3* mutants. This resulted in fluconazole resistant strains that still produced ergosterol at less than 10% of the total sterols collected. This result highlights the issue of the growth based phenotypic studies. As with the other potential growth based phenotypic studies for *erg3* yeast, these studies are an implied measure of a functional ERG3 gene [24].

These potential issues found in growth based phenotypic studies, along with the minimal amount of ergosterol found in our HPLC/MS data from ScN2 and ScN9 are the most likely reasons for the cycloheximide growth test not providing the results typical of a complemented *erg3* yeast strains. A better evaluation of the *P. capsici* DES-5 would be accomplished through the use of the yeast optimized *P. capsici* ERG3 under a strong constitutive promoter. In most of the complementation studies which used this assay, the experimental ERG3 is placed under the alcohol dehydrogenase promoter (ADH1) which is a fairly strong constitutive promoter [25]. The addition of this promoter to drive the expression of the optimized *P. capsici* ERG3 would presumably provide better expression. ScN5 and ScN8 were attempted in the cycloheximide growth assay, but conditions were never optimized.

The definitive proof that a predicted C5 desaturase is a true ERG3 enzyme is to demonstrate that ergosterol is being produced by the gene in question within the context of an

erg3 null mutant. This is not a direct measurement of the activity of an isolated enzyme, but this method has been utilized in prior studies and provides the identity of the sterol end-product, which is only possible if the pathway is truly complemented. In the present study, ergosterol production was found in HPLC/MS data from 4 separate *P. capsici* DES-5 strains (ScN2, ScN5, ScN8 and ScN9). In each case, when the Pc Erg3 protein was expressed, the correct m/z peak at the same relative retention time (RRT) of the ergosterol standard and ergosterol from the yeast control strains was observed. When the protein was not expressed, as seen with the galactose inducible strains ScN5 and ScN8, the sterol profile is the same as ScN1 KO strain. Additionally, there is an increase in ergosterol production as the amount of *P. capsici* DES-5 is increased, as seen in the Western blot data and sterol profiles from ScN5 and ScN8.

When all this data is combined, it strongly suggests that the *P. capsici* ERG3 gene is DES-5 but it is still unknown what role it plays in *P. capsici*. There has been research that demonstrates the accumulation of Δ^5 , $\Delta^{5,7}$ and Δ^7 sterols in *Phytophthora spp.* and that when absorbed they are converted to Δ^5 sterols [3, 4, 5]. It is also known that *Phytophthora* obtain all their sterols from the plants they infect through the use of elicitors. The elicitors do transport lower amounts of Δ^7 and $\Delta^{5,7}$ sterol precursors into *Phytophthora* when infecting a plant [26].

The most probable explanation for *P. capsici* possessing a DES-5 is to maximize the amount of preferred sterols, sitosterol or stigmasterol, collected by the elicitors through conversion of Δ^7 sterols and $\Delta^{5,7}$ sterols. Sterols have been proven to not be necessary for vegetative growth, but they do influence growth of *Phytophthora spp.* There is evidence to suggest that sterols are required for reproduction and that they may play a role in inducing the production of oospores. It has been shown that *P. cactorum* does not produce steroidal hormones and when incubated with only steroidal hormones, the growth pattern is similar growth

with no sterols. Some of the sterols which are absorbed are converted to esters and glycosides as seen in Nes et al (1982) paper with *P. cactorum* and cholesterol, but the majority remains as free sterols [27]. This information suggests that the sterols collected are probably used to keep the cell membrane in a liquid ordered state as is the function in most of the sterols in plants.

The source of the sterols for *Phytophthora spp* is also in a flux state depending on the season and the type of plants they use as hosts. Sitosterol, stigmasterol and campesterol are the main sterols produced in higher plants but the concentration of each varies based on the tissue of the plant, the age of the tissue, the amount of sunlight and a variety of other environmental conditions [28]. The changes seen in the sterol composition may contribute to the responses seen in *Phytophthora spp*. Previous studies evaluated the ratio of cycloartenol to sitosterol and demonstrated that a higher ratio of sitosterol creates the best vegetative growth and reproduction. A different study showed the importance of the sterol selection in creating functional membranes in oospores. Both stereochemistry of the same sterol, 17(20)-dehydrocholesterol, caused the creation of oospores but the E-17(20)-dehydrocholesterol oospores were all aborted [29]. Even the minor changes in the sterols that are absorbed by *Phytophthora spp* can play a significant role. Considering the affects the different sterols have on *Phytophthora spp* it is suspected that they also play a signaling role for different stages.

As mentioned earlier, some of the sterols that are absorbed are changed into glucosides. The cellular role of sterol glucosides has not been thoroughly investigated in the literature, but one study with cotton has provided a potential function. In this study they found Sitosterol-B-glucoside to be a regulator of cellulose synthase activity [30]. The cell wall in *Phytophthora* is composed of cellulose and it is known that sterols in Pc are converted to glucoside conjugates. Since the major sterol in *Phytophthora* is sitosterol, this could explain data from prior feeding

assays showing increased growth by providing maximal stimulation of cellulose synthase activity and hence cell wall formation.

A potential function of *P. capsici* DES-5 is to regulate the sterols absorbed. *P. capsici* have been shown to have the best vegetative growth and reproduction through the use of Δ^5 sterols. Since there are other sterol pathway precursors found in plants which are transported by elicitor there should be a method for sorting the sterols that will help promote growth from the sterols that do not promote growth. The DES-5 cannot utilize sterol substrates that lack a double bond between C7 and C8. Therefore, the sterols obtained that have not undergone Δ^8 , Δ^7 isomerization in plants are not converted by the DES-5 in *P. capsici*. Membrane sterol regulation has been shown to be a relationship between a few membrane bound enzymes (SREBP, Scap, HMG CoA reductase) and a few enzymes located in the sterol synthesis pathway [27]. Since *Phytophthora spp.* does not have a sterol biosynthesis pathway, there should be another method for regulating accumulated sterols.

In conclusion, this research has shown there is a gene in *P. capsici* that encodes DES-5 when expressed in a yeast *erg3* null mutant. Complementation of *erg3* null yeast with a *P. capsici* *ERG3* did not revert the *erg3* phenotype, but the combination of the Western blot analysis and the HPLC/MS data provide adequate support for complementation of the ergosterol pathway. The expression, cellular location and exact role of the DES-5 in *P. capsici* is still unknown, but this study provides the first evidence for *ERG3* activity from a *Phytophthora spp.* gene and provides information that suggests an abridged sterol pathway.

REFERENCES

1. Altschul, S. F., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.
2. Larkin, M.A., et al. (2001). ClustalW and ClustalX version 2.0. *Bioinformatics.* 23: 2947-2948.
3. Rahier, A. (2001). Deuterated Δ^7 -Cholesterol Analogues as Mechanistic Probes for Wild-Type and Mutated Δ^7 -Sterol-C5(6)-desaturase. *Biochemistry.* 40: 256-267.
4. Rahier, A., et al. (2000). Biochemistry and site-directed mutational analysis of Δ^7 -sterol-C(6)5-desaturase. *Biochemical Society Transactions.* 28: 799-803.
5. Taton, M., et al. (2000). Role of Highly Conserved Residues in the Reaction Catalyzed by Recombinant Δ^7 -Sterol-C5(6)-Desaturase Studied by Site-Directed Mutagenesis. *Biochemistry.* 39: 701-711, 2000
6. Sonnhammer, E. L., et al. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *In Proc. of Sixth Int. Conf. on Intelligent Systems for Molecular Biology.* 6(6912): 175-182.
7. Rahier, A., et al. (1997). The Role of Cytochrome b5 in 4 α -Methyl-Oxidation and C5(6) Desaturation of Plant Sterol Precursors. *Biochemical and biophysical research communications.* 236: 434-437.
8. Higgins, V. A., et al. (2003). Yeast genome-wide expression analysis identifies a strong ergosterol and oxidative stress response during the initial stages of industrial lager fermentation. *Applied and Environmental Microbiology.* 69(8): 4777-4787.
9. Flick, J. S., et al. (1990). Two systems of glucose repression of the GAL1 promoter in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 10(9): 4757-4769.
10. Clarke T. F., et al. (2008). Rare Codons Cluster. *PLoS ONE.* 3: e3412.

11. Bligh, E., et al. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of Biochemistry and Physiology*. 37: 911-917.
12. van West, Pieter, et al. (1999). Internuclear Gene Silencing in *Phytophthora infestans*. *Molecular cell*. 3(3): 339-348.
13. van West, Pieter, et al. (2008). Internuclear gene silencing in *Phytophthora infestans* is established through chromatin remodeling. *Microbiology*. 154(5): 1482-1490.
14. Whisson, S. C., et al. (2005). A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. *Molecular plant pathology*. 6(2): 153-163.
15. Grebenok, R. J., et al. (1998). Isolation and characterization of an *Arabidopsis thaliana* C-8,7 sterol isomerase: functional and structural similarities to mammalian C-8,7 sterol isomerase/emopamil-binding protein. *Plant Mol Biol*. 38(5): 807-15.
16. Schrick, K., et al. (2000). FACKEL is a sterol C-14 reductase required for organized cell division and expansion in *Arabidopsis* embryogenesis. *Genes Dev*. 14(12): 1471-84.
17. Darnet, S., et al. (2001). Functional identification of sterol-4 α -methyl oxidase cDNAs from *Arabidopsis thaliana* by complementation of a yeast *erg25* mutant lacking sterol-4 α -methyl oxidation. *FEBS Lett*. 508(1): 39-43.
18. Nishi, S., et al. (2000). cDNA cloning of the mammalian sterol C5-desaturase and the expression in mutant yeast. *Biochim Biophys Acta*. 1490(1-2): 106-8.
19. Husselstein, T., et al. (1999). Δ^7 -sterol-C5-desaturase: Molecular characterization and functional expression of wild-type and mutant alleles. *Plant Molecular Biology*. 39: 891-906.
20. Brumfield, K. M., et al. (2010). Functional characterization of the *Chlamydomonas reinhardtii* ERG3 ortholog, a gene involved in biosynthetic pathway. *PLoS ONE*. 5(1): e8659.
21. Iwaki, T., et al. (2008). Multiple functions of ergosterol in the fission yeast *Schizosaccharomyces pombe*. *Microbiology*. 154: 830-841.

22. Hemmi, K., et al. (1995). The physiological roles of membrane ergosterol as revealed by the phenotypes of *syr1/erg3* null mutants of *Saccharomyces cerevisiae*. *Biosci. Biotech. Biochem.* 59: 482-486.
23. Geber, A., et al. (1995). Deletion of the *Candida glabrata* ERG3 and ERG11 Genes: Effect on cell viability, Cell growth, sterol composition, and antifungal susceptibility. *Antimicrobial Agents and Chemotherapy.* 39: 2708-2717.
24. Paluszynski, J. P., et al. (2008). Genetic prerequisites for additive or synergistic actions of 5-fluorocytosine and fluconazole in baker's yeast. *Microbiology.* 154: 3154–3164.
25. Grimm, C., et al. (1991). The strong ADH1 promoter stimulates mitotic and meiotic recombination at the ADE6 gene of *Schizosaccharomyces pombe*. *Molecular and cellular biology.* 11(1): 289-298.
26. Lascombe, M., et al. (2002). The 1.45 Å resolution structure of the cryptogein-cholesterol complex: a close-up view of a sterol carrier protein (SCP) active site. *Acta Cryst.* 58: 1442-1447.
27. Nes, W. D., et al. (1982). Inhibition of growth and sitosterol-induced sexual reproduction in *Phytophthora cactorum* by steroidal alkaloids. *Phytopathology.* 72(4): 447-450.
28. Grunwald, C. (1978). Function of sterols. *Phil. Trans. R. Soc. Lond. B.* 284: 541-558.
29. Nes, W. D., et al. (1984). Side-chain structural requirements for sterol-induced regulation of *Phytophthora cactorum* physiology. *Lipids.* 19(7): 544-549.
30. Peng, L., et al. (2002). Sitosterol- β -glucoside as Primer for Cellulose Synthesis in Plants. *Science.* 295: 147-150.
31. Espenshade, P. J., et al. (2007). Regulation of sterol synthesis in eukaryotes. *Genetics.* 41: 401-427.

Appendix A

GENOMIC AND PROTEIN SEQUENCES

Phytophthora capsici ERG3 sequence

ATGAGATTGTGGAACACTTCAGCGCCATCGCGCTGCATGACAATCCGAGGCA
AGGACGGAGCTGCCAGTTTCACTTGGCAATTTCCCCCGGTTGCGAAATTTGCA
ATGGACCTCATCCTGGAATACGCCGACTACTATGCGCTAGATGCGCTGTACC
CTGCCTATCTGCCTCGTGACGACATCTACCGCCAGATTGCATCCATCACTGGA
CTTACACTGGTGGGAGGATATCTACTCTACTTCATTGGCGCTGGGTTTTCGTA
TGAATTCCTGTTCGACAAGGAGTTGATGAACCACCCCAAATTCGGAAGAAC
CAAGTACGCAGCGAAATCTTGTTCGATGAAGTCAATCCCTGGGATGGTGTCT
GTTGGCGCTCCCGTGGTTCCTTGC GGATGTCCGCGGCTTCTCCAAGACGTACA
CGGATTTTGGCAAGTTCGGCTACGGGTTTGAGGCATTGAGTGTCTGTTGCTTTC
ATTTGCTTCTCGGATATGCTGATCTACTGGTTCATCGCTGGCTACACCATCC
GCTCATCTACGCGCCACTACACAAGCCTCACCACAAGTGGATTGTCTGCTCTC
CATTTCGCATCGCACGCTTTCCACCCGGTGGACGGCTATATCCAGAGTTTGCCT
TACCATTTCTACATCATGCTCTTCCCTATCCACCGCGGTTTATTCCTCGCCCTT
TTTGTCTGCTGTGAACTTCTGGACAATCAGTATTCACGACGGATATTACCTTTC
GCACAACAAAATCGTCAATGGAGCACGACACCACTCGGTGCACCACGAACA
GTTCTGTCTACA ACTACGGGCAGTACTTCACATTCTGGGACCGAATGTTTGGAT
CCTACCGCGAACCACCTACCACAGGTTACTCCATACCCAAGGAAACATAG

Yeast Optimized *Phytophthora capsici* ERG3 Sequence

ATGGCATTATGGAACACTTCAGCCCCTAGTAGATGTATGACAATCAGAGGTA
AAGACGGTGCTGCTTCCTTCACTTGGCAATTTCCACCAGTTGCTAAGTTCGCA
ATGGATTTGATCTTGGGAATACGCCGATTATTACGCCTTAGACGCTTTGTATCC
AGCATACTTACCTAGAGATGACATCTATAGACAAATCGCTTCCATTACAGGTT
TGACCTTAGTAGGTGGTTATTTGTTGTACTTCATCGGTGCAGGTTTCGCCTAC
GAATTCTTGTTTCGATAAAGGAATTGATGAACCATCCTAAGTTTAGAAAGAATC
AAGTTAGATCCGAAATCTTGTCTCTATGAAGTCAATACCTGGTATGGTTTTG
TTAGCTTTACCTTGGTTTTTGGCAGATGTTAGAGGTTTCAGTAAAACCTATAC
TGACTTTGGTAAATTCGGTTACGGTTTTGAAGCATTATCTGTTGTAGCCTTTAT
CTGTTTCTCAGATATGTTGATCTATTGGTTTTCATAGATGGTTACATCACCCATT
GATATACGCTCCATTACATAAACCTCATCACAAAGTGGATCGTCTGCTCCCAT
TTGCTAGTCATGCATTCCACCCTGTTGACGGTTACATAACAATCTTTGCCATAC
CATTCTACATCATGTTGTTCCCTATCCACAGAGGTTTGTTTTTAGCCTTGTTTC
GTCGCTGTTAATTTTTGGACCATTTCCATACATGATGGTTACTACTTGAGTCA

CAATAAGATAGTTAACGGTGCAAGACATCACTCAGTACATCACGAACAATTC
GTCTACAACACTACGGTCAATACTTCACTTTCTGGGACAGAATGTTTCGGTTCTTA
TAGAGAACCACCTACTACAGGTTACTCAATTCCAAAGGAAACATAG

Phytophthora capsici 5-DES Sequence

MRLWNTSAPSRCMTIRGKDGAASFTWQFPPVAKFAMDLILEYADYYALDALYP
AYLPRDDIYRQIASITGLTLVGGYLLYFIGAGFAYEFLFDKELMNHPKFRKNQVR
SEILFSMKSIPGMVLLALPWFLADVRGFSKTYTDFGKFGYGFALS VVAFICFSD
MLIYWFHRWLHHLIYAPLHKPHHKWIVCSPFASHAFHPVDGYIQSLPHYFYIML
FPIHRGLFLALFVAVNFWTISIHDGYILSHNKIVNGARHHSVHHEQFVYNYGQYF
TFWDRMFGSYREPPTTGYSIPKET*

Saccharomyces cerevisiae ERG3 Sequence

ATGGATTTGGTCTTAGAAGTCGCTGACCATTATGTCTTAGACGACTTGTACGC
TAAAGTTCTGCCCGCTTCGTTGGCAGCTAATATTCCTGTCAAGTGGCAGAAAT
TGCTAGGGTTGAACAGTGGGTTTACGCAATTCTACGATTTTGCAGGAGACTTTG
AACTCCAAGAATGCCGTCAAAGAATGTAGAAGGTTCTACGGGCAGGTGCCAT
TCCTGTTTGATATGTCGACGACGTCTTTTGCATCGCTATTGCCTCGTTCAGCA
TCTTGAGAGAATTCCTCTCACTATGGGTTATTGTTACGATCTTTGGTTTACTAC
TTACTTATTACGGCTAGTCTCAGCTACGTGTTTGTGTTTGACAAGTCGATTT
TCAACCATCCTCGTTACTTGAAAAACCAAATGGCAATGGAAATCAAGTTGGC
AGTCAGTGCTATCCCATGGATGTCGATGTTGACCGTTCATGGTTTGTATGG
AATTGAACGGCCATTCTAAACTATAACATGAAGATTGATTATGAAAACCACGG
TGTAAGGAAGCTCATTATCGAGTACTTCACTTTTCATCTTTTTCACTGATTGCG
GTGTGTATTTAGCGCACAGATGGTTGCATTGGCCAAGGGTCTACCGTGCTCTG
CACAAGCCTCATCACAAGTGGCTGGTCTGCACACCTTTCGCATCTCATTCTTT
CCATCCTGTAGACGGGTTTTTGCAATCCATCTCGTACCACATCTACCCATTGA
TTCTGCCATTACACAAGGTTTCTTATTTGATTCTGTTCACTTTTGTAACTTTT
GGACTGTTATGATTCATGACGGTCAATACCTATCAAACAATCCTGCCGTCAAC
GGTACTGCCTGCCACACGGTTCACCATCTATATTTCAACTACAACACTACGGTCA
ATTCACCACTCTGTGGGACAGACTAGGGGGTTCTTACCGTAGACCAGATGAC
TCATTGTTTGATCCTAAGTTAAGAGATGCTAAGGAGACCTGGGACGCTCAAG
TTAAGGAAGTTGAACATTTTCATCAAGGAGGTGCAAGGTGATGATAATGATAG
AATCTATGAAAACGACCCAAATACCAAGAAGAACAACACTGA

Saccharomyces cerevisiae ERG3 protein sequence

MDLVLEVADHYVLDDLYAKVLPASLAANIPVKWQKLLGLNSGFSNSTILQETLN
SKNAVKECRRFYGQVPFLFDMSTTSFASLLPRSSILREFLSLWVIVTIFGLLLYLFT
ASLSYVVFVFDKSIFNHPRYLKNQMAMEIKLAVSAIPWMSMLTVPWFVMELNGH
SKLYMKIDYENHGVRLIIEYFTFIFFTDGCVYLAHRWLHWPRVYRALHKPHHK
WLVCTPFASHSFHPVDGFLQSIYHIYPLILPLHKVSYLILFTFVNFWTVMIHGQ
YLSNPAVNGTACHTVHHL YFN YNYGQFTTLWDR LGGSYRRPDDSLFDPKLRD
AKETWDAQVKEVEHFIKEVEGDDNDRIYENDPNTKKN

Appendix B

ADDITIONAL RESEARCH

In the beginning of this research (2010) the BLASTp analysis of the putative *P. capsici* *ERG3* in the *P. capsici* database provided a smaller gene sequence than what was used in this thesis. The original *Pc ERG3* was amplified out of genomic *P. capsici* DNA using the primers 1839 and 1840. This was ligated into pRS425 as described above and, after confirmation of the correct sequence, was transformed into Sc46 (wild-type yeast). The HPLC/MS data for the yeast strain containing the *Pc ERG3* under the Sc69 *ERG3* promoter, showed results similar to data from the HPLC/MS analysis of ScN2. Later in the research (2011) new data was added to the *P. capsici* database which had information suggesting more sequence up-stream and down-stream of the amplified product.

The original yeast strain used for this study was Sc46. Plasmids pDN1 and pDN2 were transformed into this strain using the methodology described above. Diagnostic PCR reactions demonstrated the transformations were correct and since these plasmids did not include a FLAG-tag, they were analyzed for functionality using HPLC/MS analysis. The HPLC/MS data was similar to what has been reported for ScN2 (*FL-PcERG3*) and ScN3 (Sc69 *ERG3*) and due to this yeast strain having normal protease function, the decision to use the Sc69 (protease deficient) yeast strain was made to help increase the protein production and therefore ergosterol production.

Originally the Sc69 *ERG3* promoter was used to drive expression of the genes in this study. Based on ergosterol production in ScN2, this promoter did not provide

adequate expression levels. Before moving to the *Gall* promoter found in ScN5, ScN6 and ScN8, a *PGK1* promoter was tested. This was amplified out of genomic Sc69 DNA using primers 1837 (TGTCGCTCGAGAAAGGAAGTGTTCCCTCCT) and 1838 (TGTCGCATATGTGTTTTATATTTGTTGTAAAAAGTAGAT) using a Tm of 50oC and an extension time of 1 minute. A 600bp PCR product was digested with *XhoI* and *NdeI* and ligated in pRS425 with the respective genes. After transformation into Sc46 and HPLC/MS analysis it was concluded that this promoter did not increase protein production when compared to using the Sc69 *ERG3* promoter. Later research provided a possible explanation to why this typically strong constitutive promoter did not increase protein production. In an article by Partow et al. from 2010 called “Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*” from the journal *Yeast*, the *PGK1* promoter which was amplified used 1000bp upstream of the *PGK1*. Potentially the promoter used in this study did not include some key components to functionality.