Structure and Function in Plant Δ12 Fatty Acid Desaturases and Acetylenases

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ABSTRACT

This study provides insight into the structure/function relationship between desaturases and acetylenases, and indicates amino acid residues within acetylenases which influence reaction outcome. Oleate desaturases belong to a family of enzymes capable of introducing *cis* double bonds between C12 - C13 in oleate esters. Acetylenases are a subset of oleate desaturase enzymes which introduce a triple bond in the C12 - C13 position of linoleate. To better understand which amino acids could be responsible for differentiating the activity of acetylenases from typical desaturases, a total of 50 protein sequences were used to compare the two classes of enzymes resulting in the identification of 11 amino acid residues which are conserved within either separate family but differ between the two groups of enzymes. These identified amino acid residues were then singularly altered by site-directed mutagenesis to test their role in fatty acid modification. Specifically, the wild type acetylenase, Crep1 from *Crepis alpina,* and a number of point mutants have been expressed in *Saccharomyces cerevisiae*, followed by fatty acid analysis of the resulting cultures. Results indicate the importance of 4 amino acid residues within Crep1 (Y150, F259, H266, and V304) with regards to desaturase and acetylenase chemoselectivity, stereoselectivity, and/or substrate recognition. The F259L mutation affected the acetylenase by converting it to an atypical FAD2 capable of producing both *cis* and *trans* isomers. The V304I mutation resulted in the conversion of Crep1 into a stereoselective FAD2, where only the *cis* isomers of 16:2 and 18:2 were produced. The Y150F mutation led to a loss of acetylenase activity without affecting the inherent desaturase activity of Crep1. The H266Q mutation appears to affect substrate selection causing an inability to bind substrate (16:1-9c and/or 18:1-9c) in a *cisoid* conformation, resulting in an increased accumulation of *trans* product. The changes in enzyme activity detected in cultures expressing Crep1 mutants demonstrate the profound effect that exchanging as little as one amino acid can have on an enzyme properties. Enzymes retain some conservation of amino acids necessary for activity, such as those involved in metal ion binding, whereas subtle changes can affect overall enzyme function and catalysis.

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DEDICATION

to my wonderful wife Christine.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

ACP	acyl carrier protein
BLASTP	basic local alignment search tool for proteins
CPDL	Conserved Property Difference Locator
ER	endoplasmic reticulum
GC-FID	gas chromatography flame ionization detection
GC-MS	gas chromatography mass spectrometer
HPLC	high-performance liquid chromatography
MMO	methane monooxygenase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NTOME	neutral theory of molecular evolution
PCR	polymerase chain reaction
PUFA	poly-unsaturated fatty acids
SCD	stearoyl-CoA desaturase
SDM	site-directed mutagenesis
TPNH	triphosphopyridine nucleotide

1. INTRODUCTION

1.1 Overview

Fatty acid desaturases are enzymes capable of modifying pre-existing carbon to carbon bonds within fatty acids. These enzymes vary in specific function and are responsible for the introduction of a wide spectrum of fatty acids found throughout nature. They are regioselective, display substrate selectivity, and introduce functionality in a stereospecific manner. One specific class of desaturases known as Δ12 fatty acid desaturases (FAD2s) modify oleic acid (*cis*-9-octadecenoic acid; 18:1–9c) by introducing a double bond between the 12^{th} and 13^{th} carbons from the fatty acid carboxyl-terminus. Other desaturase homologues have acquired diverged activity and are capable of introducing different functional groups, such as hydroxyl, epoxy, or acetylene groups. Diverged homologues that introduce a triple bond within a fatty acid are known as acetylenases.

This thesis deals with an exploration of the structure/function relationships between FAD2s and acetylenases. This introduction begins by relating a brief history of desaturases, their diversity in nature, and explains what is known of their structures and functions. This is followed by a section on evolutionarily-related variants of FAD2 desaturases, which looks more precisely at the acetylenase from *Crepis alpina,* Crep1. Previous studies that are similar in nature to this one are then discussed, in which the structural similarity of desaturases and variant homologues were considered and investigated.

1.2 Introduction to Fatty Acid Desaturases

Fatty acids are aliphatic carboxylic acids that are essential in all biological systems. They play a major role in energy storage, cell structure, cellular signaling, vitamin composition and absorption, and in metabolic regulation. They are classified based on their size and functional groups that may or may not be present within their aliphatic portion. Saturated fatty acids have no functional groups other than their carboxylic acid groups. They are made up of repeating methylene groups and are considered saturated due to the presence of hydrogen atoms occupying all carbon valencies. Unsaturated fatty acids are fatty acids which, in addition to their carboxylic acid groups, have another functional group associated with them. A good example is monounsaturated 18:1-9c which has a double bond between the 9^{th} and 10^{th} carbon positions (Figure 1.1).

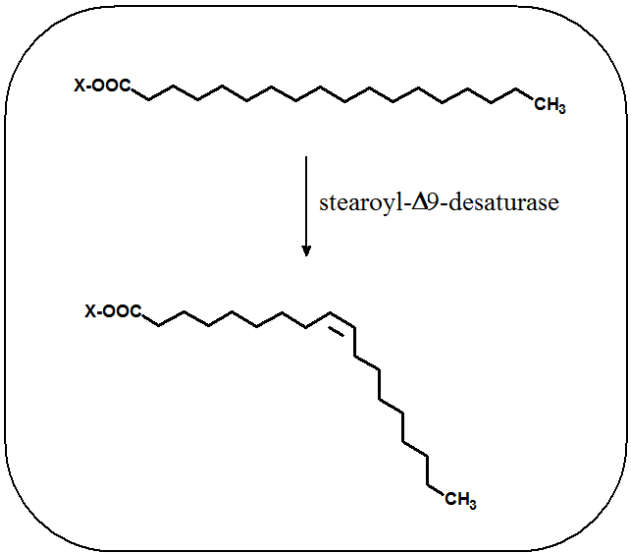

Figure 1.1 Desaturation of Stearic Acid to Oleic Acid
This reaction synthesizes oleic acid by introducing a double bond at the Δ9 position of stearic acid (18:0). In plastids, 18:0 is attached to an acyl carrier protein whereas in animals, 18:0 is attached to coenzyme A. X represents the variable substrate carrier.

1.2.1 Nomenclature

During this study the use of common names and systematic names have been simplified by using abbreviated forms which identify fatty acids by their carbon chain length, degree of desaturation, the position of the desaturation (or other functional group), and when applicable, the geometric configuration of double bonds. Figure 1.2 shows an example of this usage, linoleic acid (*cis, cis*-9,12-octadecadienoic acid) which is abbreviated to 18:2-9c,12c. Here the 18 represents the number of carbons, followed by the degree of desaturation (*i.e.* 2), followed by the position of the double bonds from the carboxylic terminus and their geometric conformation (where 9c represents the Δ9 position in the *cis* configuration and the 12c represents the Δ12 position also in the *cis* orientation). Some abbreviations do not disclose the *cis/trans* orientation, in which case the compound is assumed to always be in the *cis* orientation. Fatty acids which incorporate a different functional group are abbreviated using the same system, but replace the position of desaturation with a position of the respective functional group followed by an appropriate symbol. Examples of this are: crepenynic acid (*cis*-9-octadecen-12-ynic acid) which contains an alkyne group is abbreviated as 18:1-9c,12a;

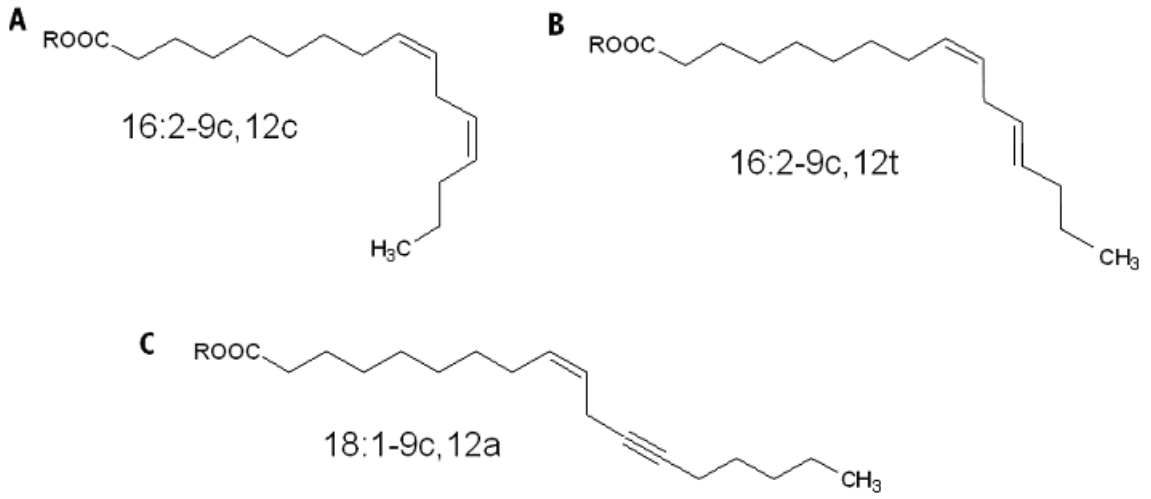

Figure 1.2 Nomenclature of Fatty Acids
Three examples are shown of fatty acid nomenclature that is used throughout this study. The first number in the names indicate fatty acid chain length, followed by the number of double bonds present, their position from the carboxyl carbon, and their geometric conformation (*i.e.* c for *cis* and t for *trans*). In the case that a different functional group is present, the position is shown followed by an appropriate symbol.16:2-9c,12c is an 16 carbon long fatty acid with 2 double bonds. One *cis* double bond is between C9-C10 and the other is between C12-C13 (A). 16:2-9c,12t is an 16 carbon long fatty acid with 2 double bonds. One is a *cis* double bond between C9-C10 and the other is a *trans* double bond between C12-C13 (B). 18:1-9c,12a is an 18 carbon long fatty acid with only one double bond, and one acetylene group (represented by the letter a). The *cis* double bond is positioned between C9-C10 and the acetylene group is found between C12-C13 (C).

vernolic acid (12,13-epoxy-*cis*-9-octadecenoic acid) is abbreviated as 18:1-9c,12e; ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) is abbreviated as 18:1-9c,12OH (Figure 1.2).

Nomenclature regarding positional regioselectivity of desaturases should also be commented on. The use of Δ assumes that a desaturase enzyme inserts a functional group by sensing the carbon position from the carboxyl terminus of fatty acids. Another method of introducing a functional group is by positioning functional groups by ω positions from the methyl end of fatty acids. Therefore ω3 fatty acid desaturases introduce a double bond 3 carbons away from the methyl end of 18:2-9c,12c, producing α–linolenic acid (18:3-9c,12c,15c) (Savile *et al.,* 2001). Other modes of desaturation include the (v+3) assignment which inserts a double bond 3 positions from an existing bond at position v (Meesapyodsuk *et al.,* 2000a).

The introduction of double bonds within fatty acids can be accomplished by enzymes known as fatty acid desaturases. There have been two groups of desaturases that have been identified; soluble (see NCBI accession #cd1050) and membrane-bound desaturases (see NCBI accession #cd01060). Although some similarities exist between these two groups, they represent two distinct families. Additionally, the more diverse membrane-bound desaturases can be further subdivided based on their particular activities and sequences homology. The variability in the activity displayed by desaturases and their homologues has led to research into how their structural composition affects overall function (Buist, 2004).

1.2.2 Survey of Fatty Acid Desaturases; History, Distribution, and Function

Unsaturated fatty acids have been detected in all forms of life although the mechanism by which their functional group is introduced is different. In anaerobic environments, prokaryotes have been shown to introduce double bonds during the biosynthesis of fatty acids. This pathway involves the dehydration of β-hydroxydecanoate (10:0-3OH), an intermediate normally involved in the biosynthesis of palmitate (16:0) and stearate (18:0). Dehydration of this substrate yields β,γ–decenoate (10:1-3c), which is then extended to form palmitoleate (16:1–9c) and vaccinate (18:1-11c) (Nagai and Bloch, 1968). However this pathway does not account for the generation of the most plentiful unsaturated fatty acid occurring in nature, 18:1-9c. As well, this pathway is only found in microorganisms and does not involve true desaturases that were later detected.

The first evidence indicating desaturation in eukaryotes was accomplished by feeding deuterium-labeled 18:0 to mice. This led to the observation that ^{2}H-18:0 was directly converted to ^{2}H-18:1-9c acid (Schoenheimer and Rittenberg, 1935). It is now known that the synthesis of 18:1-9c is an aerobic process achieved in eukaryotes and is the preliminary desaturation step involved in further production of polyunsaturated fatty acids.

It was not until the 1960s that research involving phytoflagellate *Euglena gracilis* showed that a cell-free soluble desaturase system could be reconstituted when 3 components were brought together. It was revealed that 18:0 could be desaturated to 18:1-9c when (i) a flavin portion capable of oxidizing triphosphopyridine nucleotide (TPNH), an electron acceptor, was added with (ii) a non-heme iron protein which behaved like ferredoxin, where it acts in concert as an electron transport chain for (iii) a particulate fraction containing an

unidentified desaturase enzyme (Bloomfield and Bloch, 1960). This discovery allowed for more focused research into desaturases and led to the identification and isolation of the three enzyme components required (Holloway *et al.,* 1963; Gelhorn and Benjamin, 1964; Oshino *et al.,* 1966; Gurr *et al.,* 1970; Holloway and Wakil, 1970; Oshino *et al.,* 1971; Oshino and Sato, 1971; Spatz and Strittmatter, 1971; Holloway and Katz, 1972; Shimakata *et al.,* 1972; Strittmatter *et al.,* 1972). Two of the identified components are required in the electron transport chain involved in desaturation and will be discussed later. The last component isolated was the terminal component in the electron transport chain, the stearoyl-CoA desaturase (SCD) from rat (Strittmatter *et al.,* 1974). The isolation of rat SCD allowed for its cDNA to be characterized and cloned (Thiede *et al.,* 1986), which paved the way for other desaturases to be characterized in subsequent research. Similar desaturases were identified in other organisms including mouse (Ntambi *et al.,* 1988), yeast (Stukey *et al.,* 1989), and humans (Li *et al.,* 1994; Zhang *et al.,* 1999).

Research generated from earlier definitions of desaturase systems did not solely revolve around animal systems. Many studies in plant systems were also conducted, which eventually led to the purification of a soluble stearoyl-acyl carrier protein (ACP) Δ9-desaturase from safflower (McKeon and Stumpf, 1982). Later studies allowed for the full nucleotide sequence of stearoyl-ACP Δ9-desaturases from castor and cucumber to be reported (Shanklin and Somerville, 1991). These soluble desaturases, which are only found in the plastids of plants, are not evolutionarily related to the membrane-bound desaturases, which are found in all eukaryotes. Membrane-bound desaturases are not soluble and are instead found integrated within the endoplasmic reticulum and plastid membranes.

Although much research generated information on desaturase system components and allowed for the identification of many desaturases, all the desaturases discussed so far are responsible for the introduction of double bonds at the Δ9 position within unsaturated fatty acids. These desaturases do not explain desaturation which occurs at other locations, such as those involved in the generation of poly-unsaturated fatty acids (PUFAs). In the late 1980s, a large study considering lipid metabolism in plant systems was conducted and led to the identification of mutants in *Arabidopsis thaliana* plants that resulted in modified lipid pools (Browse *et al.,* 1985; Feldmann *et al.,* 1989; Somerville and Browse, 1991). This research indicated the existence of many desaturases in plants, which exist in specific cellular

compartments, and differentially regulate lipid metabolism. One such mutant which failed to accumulate 18:2–9c,12c allowed for the identification and cloning of the Δ12 fatty acid desaturase from *Arabidopsis* (Okuley *et al.,* 1994). FAD2 now represents a class of fatty acid desaturases responsible for the generation of 18:2–9c,12c from its precursor 18:1-9c (Figure 1.3). FAD2 is an integral membrane-bound protein and is not related to the soluble stearoyl-ACP Δ9-desaturase. In addition to *fad2*, this research also allowed other plant desaturase genes to be identified including *fab*2, *fad*3, *fad*4, *fad*5, *fad*6, *fad*7, and *fad8* (Miquel and Browse, 1992; Miquel *et al.,* 1998).

It is now recognized that fatty acid desaturases make up a broad group of enzymes that carry out a large number of reactions on various substrates. Two distinct groups of fatty acid desaturases exist: one smaller family found only in plants consists of the soluble acyl-ACP desaturases belong to the ferritin-like superfamily; the other is a larger and more diverse superfamily of membrane bound desaturases which have been detected in a range of organisms including fungi, plants and animals (Shanklin and Cahoon, 1998). The soluble desaturases are not evolutionarily related to membrane bound desaturases and carry out desaturation reactions on newly generated acyl-ACP substrates found exclusively in the plastids of plants. In general these reactions introduce a *cis* double bond at the Δ9 position of 18:0, although some members of this family display variability in their regioselectivity and in their substrate selectivity. Some examples of these variant acyl-ACP desaturases include myristoyl-Δ9-acyl-ACP desaturase from *Asclepius* (Cahoon *et al.,* 1997a), palmitoyl-Δ5-acyl-ACP desaturase from *Basia* (Whitney *et al,.* 2000), and palmitoyl-Δ6-acyl-ACP desaturase from *Thunbergia* (Cahoon *et al.,* 1994).

The second family of desaturases consists of a much larger group of membrane bound enzymes which catalyze a wider range of reactions than those seen in the soluble acyl-ACP desaturases. These reactions include *cis* and *trans* desaturation, hydroxylation, epoxidation, acetylenation, and are suspected to even carry out aldehyde decarbonylation and ubiquinol dehydrogenation (Shanklin and Cahoon, 1998). The substrates upon which these enzymes carry out these reactions are not limited to saturated fatty acids, but include mono and polyunsaturated fatty acyl esters, aldehydes, sphingolipids, sterols, and carotenoids. In recent work (Sperling *et al.,* 2003) the phylogenetic relationship of desaturases allowed for the further

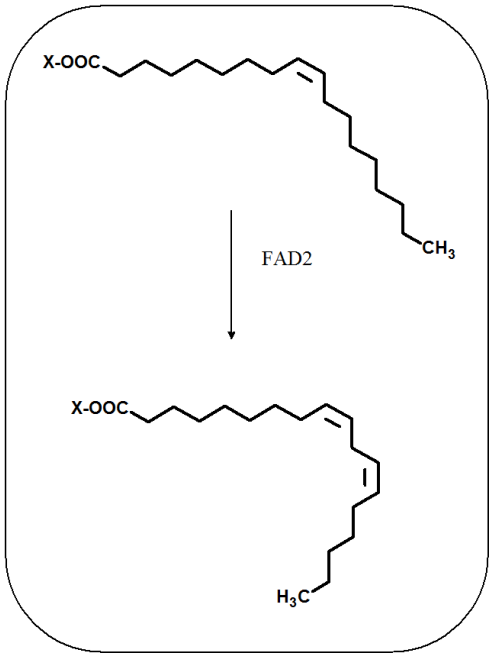

Figure 1.3 Desaturation of Oleic Acid to Linoleic Acid
This reaction is enzymatically catalyzed by membrane-bound fatty acid desaturases (FAD2). Linoleate (18:2–9c,12c) is produced after the introduction of a double bond at the Δ12 position of oleate (18:1–9c). Substrate is attached to the *sn2*-position of phosphatidylcholine indicated by the X position.

sub-classification of membrane desaturases based on their sequence similarity and function. This work shows that the differences in the spacing of amino acid recognition sequences found in all membrane bound desaturases may be used to divide this family into two subclasses. Further categorization of desaturases has also been accomplished by sub-dividing the desaturases according to their function and phylogeny (Figure 1.4). Sperling *et al.,* 2003, divided membrane bound desaturases into the following groups: i) desaturases that introduce a double bond in saturated substrates (*i.e.* Δ5, Δ7, Δ9, and Δ11 desaturases), ii) sphingolipid modifying enzymes (*i.e.* Δ4E, Δ2OH, Δ4OH, and Δ8(E/Z)), iii) Δ4, Δ5, Δ6,and Δ8 front-end desaturases (*i.e.* desaturases capable of inserting a double bond between preexisting double bonds and the carboxyl terminus of a fatty acid), and iv) Δ12/ω6, Δ15/ω3 desaturases and their exotic paralogues (where Δ defines the position of a *cis* double bond unless followed by E which defines a *trans* double bond, or OH which represents hydroxylation, or E/Z defines a bifunctional *cis/trans* desaturase). A similar division of desaturases based on their phylogeny

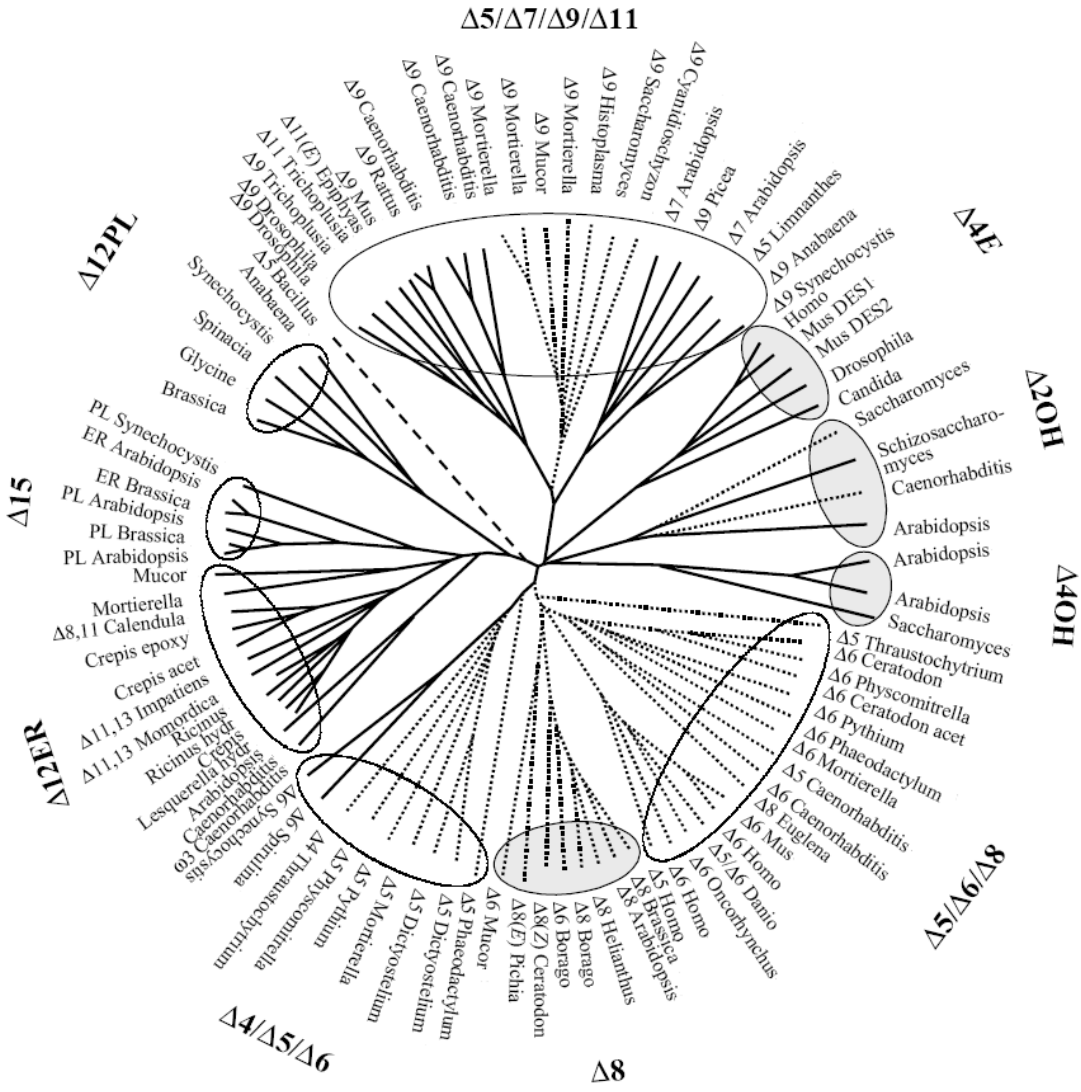

Figure 1.4 Regioselectivity Phylogram of Lipid Desaturases and Modifying Enzymes
This diagram is reproduced from Sperling *et al.* 2003. The programs CLUSTALX and TreeView were used to align and group full-length amino acid sequences in a radial diagram according to their regioselectivities. Regioselectivities are symbolized by numbers (Δ-desaturases) and subcellular localization (PL=plastidial, ER=microsomal). Sphingolipid acyl amide α-hydroxylases and sphingolipid sphingoid C4-hydroxylases are designated as Δ2OH and Δ4OH. Sphingolipid sphingoid desaturases are represented by Δ4E and Δ8. A grey background indicates sphingolipid-modifying enzyme groups. Dotted branches represent cytochrome b5 fusion proteins. Variant exotic modifications (epoxidation, hydroxylation, conjugation and acetylenation) are noted behind the organism. References for sequences are found in the original work. Reproduced with permission from Sperling *et al.,* 2003.

and function has also been shown in work conducted strictly on membrane bound desaturases (Alonso *et al.,* 2003). Their work allowed for the partitioning of desaturases into three clusters consisting of the Δ9 desaturases, front-end desaturases (namely Δ5, Δ6 and Δ8 desaturases), and Δ12/ω6 and Δ15/ω3 desaturases. Although the study by Alonso *et al.* (2003) considers a smaller range of desaturases than Sperling *et al.* (2003), the results are in congruence with each other, validating the sub-categorization of membrane bound desaturases based on their phylogenic roots and their function.

Front-end desaturases are required for the synthesis of PUFAs but are not reviewed in this work. Additional information regarding front-end desaturases and their role in the synthesis of PUFAs can be found in other reviews (Pereira *et al,.* 2003; Napier *et al.,* 2003; Nakamura and Nara, 2004).

1.2.3 Structure of Fatty Acid Desaturases and Their Components

The main focus of this section will be microsomal FAD2s and related enzymes, and their electron transport components. Although soluble desaturases are not directly related with this study, their structure and components are briefly discussed followed by a description of membrane-bound desaturase systems. A great deal of information on soluble desaturases can be found in an elaborate review by Shanklin and Cahoon , 1998.

1.2.3.1 Soluble Desaturase Systems

The amino acid sequences derived from soluble desaturases place them in a separate family as those found in membrane-bound desaturases. Differences existing in amino acid motifs that are believed to co-ordinate a diiron cluster separate the soluble desaturases from the membrane bound desaturases. Soluble desaturases contain two amino acid sequence motifs (D/EXXH) which act as ligands for the diiron cluster that is necessary for enzyme activity. These motifs are not found in membrane-bound desaturases, which instead contain three conserved histidine boxes that are thought to function in an analogous fashion as the two D/EXXH motifs found in soluble desaturases. Soluble desaturases are exclusively localized in plastids where they carry out reactions on saturated acyl-ACP substrates.

Although electron transport components for soluble desaturases are similar to microsomal systems some differences exist. In non-photosynthetic tissue, soluble desaturases

receive their reducing equivalents indirectly from nicotinamide adenine dinucleotide phosphate oxidase (NADPH). The transfer of electrons from NADPH is achieved by ferredoxin-$NADP^+$ oxidoreductase flavoprotein which then passes the electrons to the 2Fe-2S ferredoxin electron carrier and then to the soluble desaturase itself. In photosynthetic tissue, no NADPH or flavoprotein is required since reducing equivalents are generated from photosystem I and transferred to ferredoxin which directly supplies the desaturases (Jacobson *et al.,* 1974).

The over-expression of soluble stearoyl-ACP Δ9-desaturase has led to its isolation and allowed for its biochemical characterization (Thompson *et al.,* 1991). The inclusion of a diiron cluster has been confirmed and is believed to be directly involved in catalysis (Fox *et al.,* 1993). The Δ9-stearoyl-ACP desaturase from castor has been isolated and crystallized (Lindqvist *et al.,* 1996) revealing information on the Fe-ligand properties as well as a hydrophobic channel presumed to be the substrate binding pocket. A consensus motif $[(D/E)X_2H]_2$ involved in the binding of the metal cofactors has been identified, allowing further detection of a number of enzymes believed to be related to soluble desaturases, including a phenol hydroxylase from *Pseudomonas*, an alkene monooxygenase from *Mycobacterrium* and methane monooxygenase from *Methylococcus* (Shanklin and Cahoon, 1998).

1.2.3.2 Electron Transport in Membrane-Bound Desaturase systems

Studies conducted in the 1960s identified 3 cellular components in protozoans as a requirement for successful desaturation in nature (Nagai and Bloch, 1968). These microsomal components are cytochrome b_5 reductase, cytochrome b_5, and a terminal desaturase enzyme (Figure 1.5). In addition to these components it was also shown that the coenzyme nicotinamide adenine dinucleotide (NADH) and oxygen are required for desaturase activity. The requirement of oxygen for successful desaturation in eukaryotes has been known for a long time. It was initially reported that yeast could not be cultured under anaerobic conditions (Pasteur, 1879) but was at a later date shown that yeast could be cultured if the media was supplemented with 18:1-9c and ergosterol (Andreassen and Stier, 1954). These early observations were indicators that oxygen was a requirement for desaturase activity. It is now known that oxygen serves as the terminal electron acceptor in desaturase reactions (Goldfine, 1965).

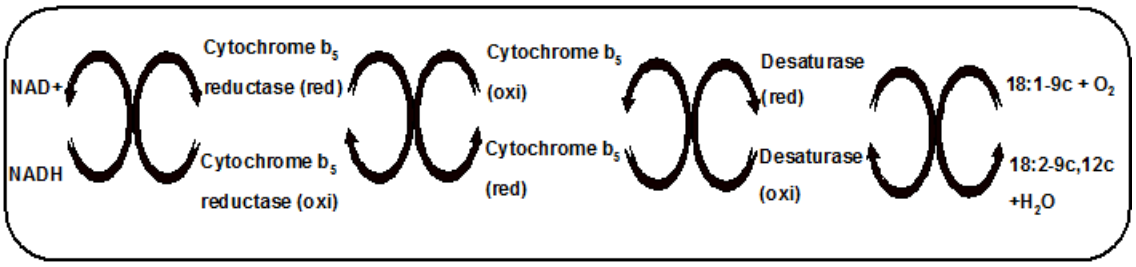

Figure 1.5 Electron Transport Chain Components for Microsomal Desaturases
The flow of electrons originates at the NADH (left side) and is passed down to the terminal desaturase enzyme through a series of reactions involving cytochrome b_5 reductase and cytochrome b_5. The final products include water (reduced from molecular oxygen) and linoleate (from oleate) esterified at the *sn2*-position of phosphatidylcholine.

Cytochrome b_5 reductase is the first electron transport component involved in the electron transfer from NADH to the desaturase enzyme. This reductase is an amphipathic flavoprotein consisting of a hydrophobic portion which attaches itself to the endoplasmic reticulum (ER) membrane, and a hydrophilic section capable of interacting with the aqueous cytosol and polar head groups of phospholipids. The hydrophobic portion of cytochrome b_5 reductase is required for the anchoring of cytochrome b_5 to the microsomal membrane (Strittmatter, 1972).

Cytochrome b_5 is a hemoprotein acting as an electron transport intermediate responsible for the delivery of electrons from cytochrome b_5 reductase to microsomal desaturases. The cytochrome involved in desaturation is the microsomal form of cytochrome b_5. The crystal structure for bovine cytochrome b_5 has been resolved and shows an alpha+beta class fold capable of supporting a central heme group (Argos and Mathews, 1975). Microsomal cytochrome b_5 also has a 40 amino acid hydrophobic segment believed to interact with the ER membrane and with cytochrome b_5 reductase (Spatz and Strittmatter, 1971). Front-end desaturases exist as fusion proteins which incorporate the cytochrome b_5 component to either the amino or carboxyl terminus of the desaturase enzyme (Napier *et al.,* 2003). Electron transport and the required components for FAD2 desaturation are shown in Figure 1.5.

1.2.3.3 Integral Membrane Desaturases

The structure of membrane desaturases is not known since these enzymes are recalcitrant to purification due to their lipophilic nature. Although numerous attempts at purifying microsomal

desaturases have yielded some results (Strittmatter *et al.,* 1972), a crystal structure has yet to be resolved. Despite this obstacle, genomic information obtained since the first sequenced FAD2 has revealed much about FAD2s and similar enzymes (Somerville and Shanklin, 1996). Membrane-bound desaturases can now be recognized by a conserved signature sequence found in all these desaturases (Stukey *et al.,* 1989). Three conserved histidine sequences (known as His-boxes) with the motifs $[HX_{(3-4)}H]X_{(7-41)}[HX_{(2-3)}HH]X_{(61-189)}$ $[(H/Q)\ X_{(2-3)}HH]$ have been identified in all integral membrane desaturases and are believed to coordinate a diiron-oxygen cluster which makes up the catalytic site of the enzymes. The idea that the diiron cluster makes up the enzyme active site is supported by the fact that replacing any of the conserved histidine residues within these motifs ablates enzymatic activity in desaturases (Shanklin, 1994; Avelange-Macherel and Macherel, 1995). Variability existing in the space between the second and the third His-boxes, without affecting the hydrophobic segments found in between, has allowed the separation of membrane bound desaturases into two groups. One group of desaturases contains the shorter sequences and is believed to have evolved from the 'longer' sequence desaturases via gene deletion. The longer sequence group of desaturases contains all acyl and sphingolipid modifying desaturases, whereas the shorter sequence group includes only one known formal desaturase (a sterol Δ5 desaturase) and is made up primarily of sterol hydroxylases (Sperling *et al.,* 2003).

1.2.3.4 Desaturases Contain Iron

The presence of iron within desaturases has been known since the isolation of rat stearoyl-CoA Δ9-desaturase. It was first assumed that only one atom of non-heme iron was present per desaturase protein (Strittmatter *et al.,* 1974), but it is now known that two non-heme iron ions are integrated within desaturases (Broadwater *et al.,* 1998). The diiron cluster is required for activity and believed to be part of the active site within desaturases and related enzymes (Shanklin *et al.,* 1997). Mechanisms involving similar diiron clusters have been based on similar systems for methane monooxoygenase (MMO) (Shu *et al.,* 1997) and more recently on *Pseudomonas oleovorans* alkane ω-hydroxylase (AlkB) (Shanklin and Whittle, 2003). A number of soluble diiron proteins classes have been identified. Class I includes hemerythrin and myohemerythrin, coordinated by nitrogen ligands (Holmes *et al.,* 1991). Class II includes soluble acyl-ACP desaturases, ribonucleotide reductase and MMO coordinating the diiron

cluster via oxygen ligands (Wallar and Lipscomb, 1996; Fox *et al.,* 1994; Kurtz, 1990). Class III includes variant binding motifs for the diiron cluster and include the membrane bound desaturases and AlkB (Shanklin and Whittle, 2003). These kinds of metalloenzymes encapsulate two iron ions within a coordination environment involving histidine nitrogen bridged to oxygen. The assumption that conserved His-boxes coordinate a metal active site is supported by topological models (Figure 1.6) of integral membrane desaturases that place all the His-boxes on the cytoplasmic face of the ER membrane (Stuckey *et al*., 1990; Shanklin *et al.,* 1994; Man *et al.,* 2006).

1.2.3.5 Mechanism of Desaturation

The mechanism by which desaturation occurs has been well-studied over the last decade. Desaturation is a positional and geometrically stereospecific reaction which involves the (slower) abstraction of one hydrogen followed by the (faster) abstraction of another hydrogen from a neighboring carbon. Evidence for this mechanism has been shown as early as the mid century involving Δ9 desaturation. Investigators used four stereospecifically labeled ^{3}H-18:0 incubated with *Corynebacterium diphtheria* cultures to show that the pro-R tritium was lost in Δ9 and Δ10 ^{3}H-18:0, but not with pro-S hydrogen. Isotope effects also allowed investigators to suggest that the formation of 18:1-9c was preceded by the removal of hydrogen at the Δ9 position of 18:0 followed by another hydrogen at the Δ10 position (Schroepfer and Bloch, 1965a; Schroepfer, 1965b). Kinetic isotope effects have also been used to assess the mechanism of Δ12 desaturation which indicates an early hydrogen abstraction from 12th carbon followed by a fast hydrogen abstraction at the 13th carbon (Behrouzian and Buist, 1998). It is now believed that a diiron-oxo cluster ligated to conserved amino acids within desaturase enzymes make up the active site (Fox *et al.,* 1993). Currently our understanding of iron's role during desaturation is thought to be similar to that of soluble MMO (Shu *et al.,* 1997). At rest, MMO's diiron core is in the oxidized diferric (Fe^{III}- Fe^{III}) form. The reduction of this core is accomplished by NADH resulting in the diferrous (Fe^{II}-Fe^{II}) form. Molecular oxygen can then bind the diferrous center to produce a peroxo form (P) which is then converted to an active form (Q) capable of abstracting hydrogen from the substrate. The substrate is converted to an intermediate radical, which is quenched by the caged hydroxyl group, resulting in a hydroxylated product and the recycling of the diiron core back to the diferric form. The

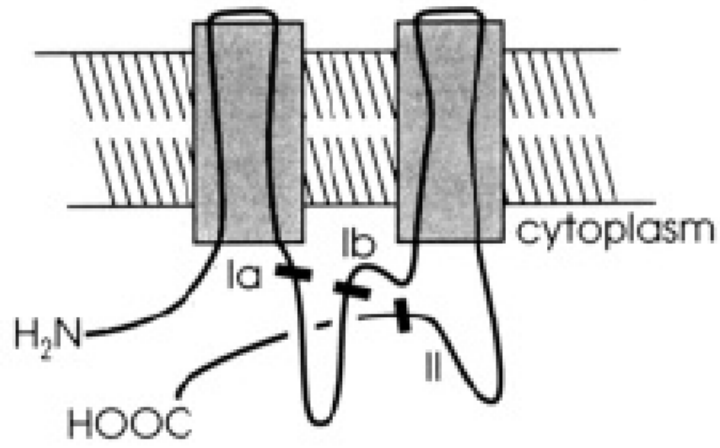

Figure 1.6 Proposed Topology of FAD2 Desaturases
Several desaturases predicted by Shankin *et al.* 1994 all retain the same topology predicting that the three conserved histidine-rich motifs are found in the cytoplasmic face of the endoplasmic reticulum. In their diagram Ia, Ib, and II represent the histidine-rich motifs (His boxes) which are alleged to coordinate an oxygen containing diiron cluster. This figure is reproduced from Shankin *et al.* 1994.

role of iron within desaturases is believed to act in the same way, although no hydroxyl group is returned to the substrate, and instead another hydrogen is abstracted allowing a diene to be created. Hydroxyl groups which are bound to the diiron cluster are then believed to be released as water, which recycles the diiron center back to its original form (Shanklin and Cahoon, 1998) (Figure 1.7). A much more definitive explanation of the mechanisms involved during desaturation amongst the many different desaturases has been well reviewed by Buist, 2004.

Substrate also plays an important role in desaturation since specificity of desaturases is not usually very permissive. Beyond simply recognizing chain length and the degree of saturation, desaturases require that fatty acids are introduced by specific carriers. Phospholipids have been successfully demonstrated as being a substrate for membrane-bound desaturases and homologs. Thus it is now recognized that FAD2s preferably act on 18:1-9c residues at the *sn*2 position of phosphatidylcholine (Sperling *et al.,* 1993; Sperling and Heinz, 1993).

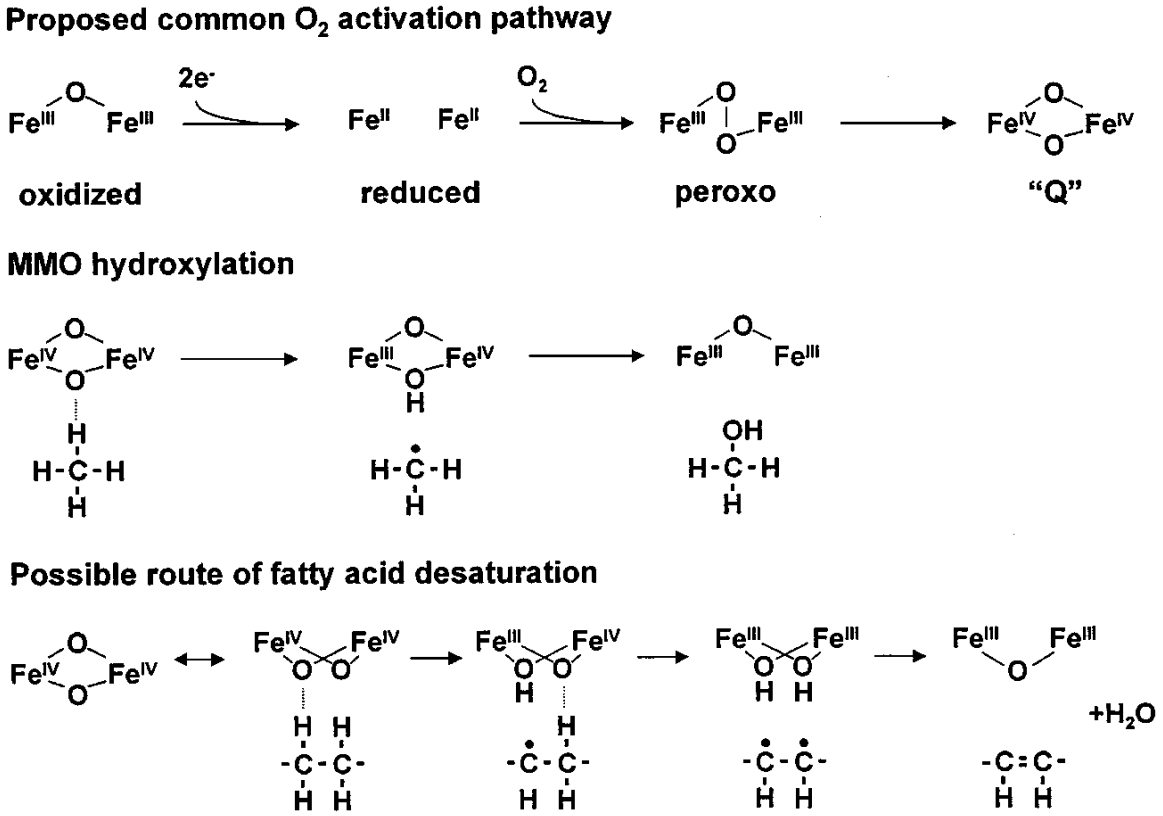

Figure 1.7 Proposed Mechanisms for MMO and Desaturases
This diagram from Shanklin and Cahoon, 1998, is a proposed mechanism of desaturation which considers the activation states of the diiron cluster. Top panel, proposed common oxygen activation pathway; middle panel, MMO proposed reaction path; lower panel, possible route of fatty acid desaturation. Reproduced from Shanklin and Cahoon, 1998.

1.3 Variants of FAD2 Desaturases

The conserved motif for membrane-bound desaturases have allowed for the identification of many proteins which make up a super-family of non-heme oxygen dependent enzymes. These enzymes include the Δ9-desaturases isolated from rat and yeast (OLE1) responsible for the production of 18:1-9c from 18:0, FAD2s responsible for the introduction of a double bond at the Δ12 position of 18:1-9c during synthesis of 18:2–9c,12c, and FAD3s responsible for the introduction of a double bond at the Δ15/ω3 position of 18:2–9c,12c. Other FAD2-related enzymes which introduce different functional groups within fatty acids have also been included within this super family. This section begins by discussing some of the variant desaturases and continues to elaborate on a specific FAD2 variant from *Crepis alpina,* the acetylenase Crep1.

1.3.1 Chemoselectivity of FAD2 Variants

A number of microsomal desaturase-related enzymes are highly similar to FAD2s do not primarily introduce double bonds. Instead these enzymes are capable of introducing various functional groups at select positions within fatty acids (Figure 1.8).

Some of these enzymes such as the fatty acid hydroxylase from *Ricinus communis* (castor) introduce a hydroxyl group at the Δ12 position of 18:1-9c (Van de Loo *et al.,* 1995). This hydroxylase is believed to be evolutionarily derived from membrane-bound desaturases, sharing a 67% sequence similarity with *A. thaliana* FAD2. The requirement of non-heme iron by this enzyme was suggested after observations that activity was reduced in the presence of cyanide, azide, or metal chelators, but was not affected by carbon monoxide (Galliard and Stumpf, 1966).

Another FAD2 variant cloned from *Lesquerella fendleri* has been shown to be bifunctional as a hydroxylase and a desaturase (Broun *et al.,* 1998a). This enzyme was isolated on the basis of nucleotide sequence similarity with the *R. communis* Δ12 hydroxylase and expressed in *Arabidopsis thaliana*. It was shown that 18:1-9c,12OH did accumulate in the seeds of transgenic *A. thaliana.* Furthermore, when this same enzyme was expressed in *A. thaliana fad2-3* mutants, which have reduced capability in producing 18:2-9c,12c, the enzyme was shown to function as an oleate Δ12 desaturase. This is not the case with the *R. communis* hydroxylase which was also examined in parallel experiments which examined fatty acid accumulation, but did not differ significantly from untransformed plants. Other FAD2 variants known as epoxygenases from *Vernonia galamensis* (Ironweed) (Hitz, 1998) and *Crepis palaestina* (a relative of dandelion) (Lee *et al.,* 1998) have been shown to introduce epoxy groups instead of double bonds within fatty acids. The expoxygenase from *C. palaestina* was cloned from developing seeds and shown to encode a protein of 374 amino acids. This enzyme shares sequence similarity with the *Arabidopsis* Δ12 desaturase (58% identity) and the *R. communis* hydroxylase (53% identity). Transgenic expression of *C. palaestina* epoxygenase resulted in the accumulation of vernolic acid (18:1-9c,12e) in *A. thaliana* seeds to 15% (w/w) of total fatty acids.

Conjugated trienoic systems have also been found to be introduced by FAD2 related enzymes. Conjugated systems differ from methylene interrupted diene systems since double bonds exist without methylene interruption (Figure 1.8). FAD2-related conjugases from

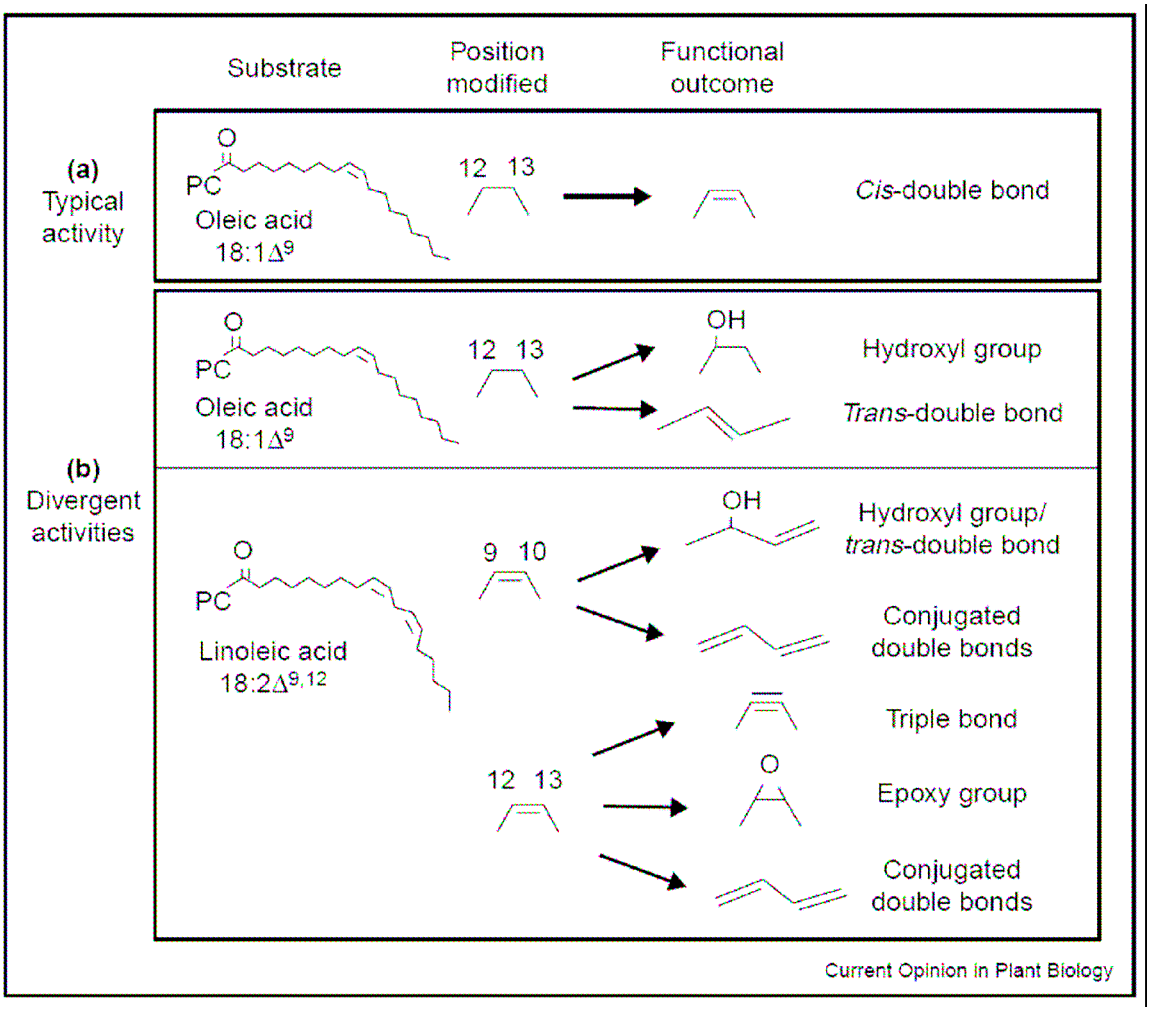

Figure 1.8 FAD2 and FAD2 Variant Reactions
This figure lists the typical activity for FAD2 and alternative reactions from FAD2 divergents. (a) FAD2 typically introduces a *cis* double bond at the Δ12 position of 18:1-9c bound to phosphatidylcholine. (b) Alternative reactions in FAD2 homologs responsible for the introduction of other functional groups such as hydroxyl groups, conjugated bonds, triple bonds, and epoxy groups. Reproduced from Jaworski and Cahoon, 2003.

Calendula officinalis (marigold) and *Mormordica charantia* (bitter melon) that are responsible for the modification of Δ9 carbons during the synthesis of calendic acid (18:3-8t,10t,12c) have also been reported (Cahoon *et al.,* 1999; Fritsche *et al.,* 1999; Cahoon *et al.,* 2001). By using an expressed sequence tag approach, two enzymes from *Calendula officinalis* were isolated. These enzymes shared 40-50% amino acid identity with other known FAD2s. Transgenic expression of these enzymes in soybean embryos and in yeast supplied with 18:2–9c,12c resulted in the accumulation of calendic acid.

Two variant FAD2 enzymes (DsFAD2-1 and DsFAD2-2) have been isolated and cloned from *Dimorphotheca sinuata* and found to be involved in the synthesis of dimorphecolic acid (18:2-9OH,10t,12t). Expression of DsFAD2-1 in yeast and soybean embryos resulted in the accumulation of 18:2-9c,12t instead of its geometric isomer, 18:2-9c,12c. 18:2-9c,12t was then further modified to dimorphecolic acid by DsFAD2-2. When DsFAD2-2 was expressed in yeast without the 18:2-9c,12t substrate and in the absence or presence of a typical FAD2, a dimorphecolic isomer was produced (18:2-9OH,10c,12c). These results imply that DsFAD2-2 is a bifunctional FAD2-variant capable of inserting a hydroxyl group at the Δ9 position with a concordant double bond migration (Cahoon and Kinney, 2004).

FAD2 related acetylenases which introduce an acetylene bond at the Δ12 position of 18:2-9c,12c bound to the *sn2*-position of phosphatidylcholine have been cloned from *Crepis alpina* (Lee *et al.,* 1998) and *Petroselinum crispum* (parsley) (Cahoon *et al.,* 2003). Crep1, an acetylenase from *C. alpina* was the first functional acetylenase to be cloned. Cloning was achieved by using cDNA from *C. alpina* developing seeds and amplifying the gene by PCR using degenerate FAD2 and castor hydroxylase primers. The protein was shown to share 59% amino acid identity with *R. communis* hydroxylase and 56% amino acid identity with *A. thaliana* FAD2. Crep1 was expressed in *S. cerevisiae* in the presence of 18:2–9c,12c, which allowed for the production of 18:1-9c,12a.

The up-regulation of FAD2-like enzymes in parsley after being exposed to a peptide fungal elicitor allowed for the detection of ELI12 (Kirsch *et al.,* 1997). In later studies, the expression of ELI12 in transgenic soybean embryos led to the accumulation of 18:1-9c,12a, defining ELI12 as an FAD2-derived acetylenase (Cahoon *et al.*, 2003). Furthermore, this work also led to the identification of several acetylenases in other members of the Apiaceae, Asteraceae, and Araliaceae families.

Another variant of FAD2 enzymes is the more recently isolated *Cop-odeA* desaturase from the fungus *Coprinus cinereus* (Zhang *et al.,* 2007). This enzyme has been shown to function as a bifunctional Δ12/Δ15 desaturase after being expressed in *S. cerevisiae* and in which total fatty acids comprised of 8.8% 16:2-9c,12c, 1.0% 16:3-9c,12c,15c, 29% of 18:2-9c,12c and 0.6% 18:3-9c,12c,15c. Similar Δ12/Δ15 fatty acid desaturases have also been reported in other filamentous fungi (Damude *et al.,* 2006).

Of course these variants are just some examples of the many bifunctional and exotic desaturases that have been reported in nature. Many other examples of FAD2 variants exist. Additionally, the bifunctionality of desaturases is also believed to be an endogenous trait of most desaturases to some degree. The degree of functionality is believed to be determined by specific amino acid residues found near the active site or acyl-binding pocket of desaturases (Buist, 2004).

1.3.2 An Acetylenase from *Crepis alpina,* Crep1

During analytical investigations of *Crepis foetida* seed oil, a novel fatty acid *cis*-9-octadecen-12-ynoic acid (18:1-9c,12a) (Figure 1.9) was discovered (Mikolajczak *et al.,* 1964). This compound which made up 60% of the total oil content in seeds was named crepenynic acid (18:1-9c,12a). Some early attempts in understanding the biosynthesis of 18:1-9c,12a used radioactive labeling experiments allowing researchers to suggest that the acetylene was introduced in pre-existing unsaturated fatty acid. This research also indicated that 18:1-9c,12a residues were found predominantly in the *sn*2 and *sn*3 position of triacylglycerols in developing seeds (Haigh *et al.,* 1968). 18:1-9c,12a was also detected in other plants such as in *Afzelia cuanzensis* (Gunstone *et al.,* 1967) and *Ixiolaena brevicompta* (Ford *et al.,* 1983) and received more attention after being linked to mortality in sheep (Ford *et al.,* 1986). Toxic effects of 18:1-9c,12a are now believed to be due to its interfering effect during prostaglandin and leukotriene synthesis (Ford *et al.,* 1986; Croft *et al.,* 1987; Nugteren and Christ-Hazelhof, 1987). 18:1-9c,12a has also been implicated as a precursor in the production of falcarinol (Barley *et al.,* 1988), a naturally occurring fungicide with reported anti-carcinogen activity (Zheng *et al.,* 1999; Kobaek-Larsen, 2005). Work conducted by Johansson and Samuelsson, 2001, provided further physical and chemical properties of 18:1-9c,12a as compared to 18:1-9c and 18:2-9c,12c esters. Their work showed that 18:1-9c,12a does oxidize extremely rapidly.

More definitive studies conducted in the late 1990s allowed researchers to conclude that a Δ12 acetylenase was responsible for the introduction of the acetylene group in 18:1-9c,12a (Lee *et al.,* 1998). The substrate involved during the production of 18:1-9c,12a was identified as 18:2-9c,12c by using radio-labeled substrates. The responsible gene (*crep1*) was cloned using primers designed for endoplasmic reticular desaturases and cDNA from *Crepis*

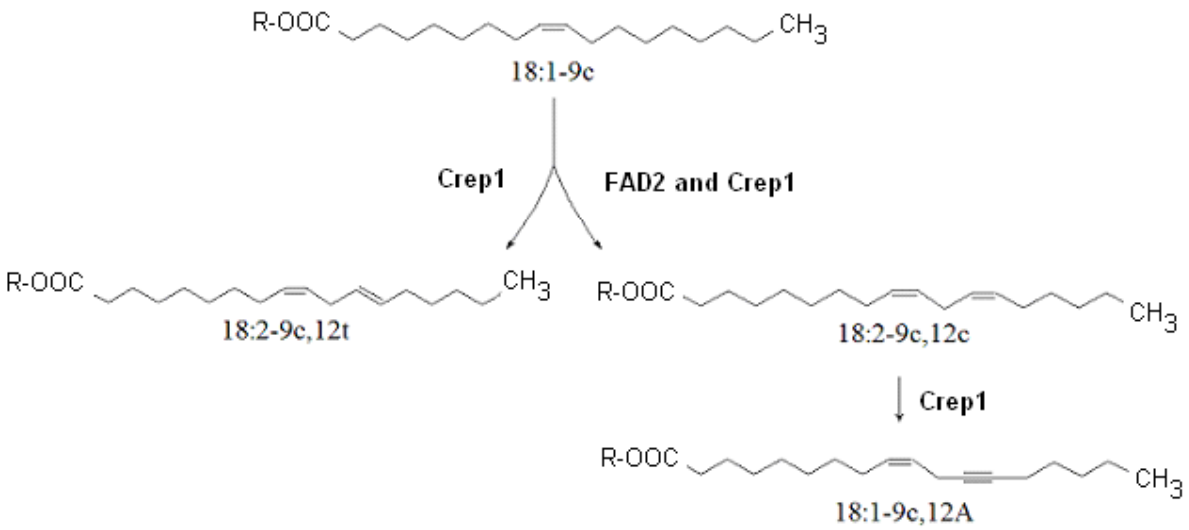

Figure 1.9 Reactions Catalyzed by Crep1
Crep1 is a bifunctional desaturase/acetylenase acting on (i) oleic acid (18:1-9c) to produce 18:2 geometric isomers (18:2-9c,12c and 18:2-9c,12t), and on (ii) linoleic acid (18:2-9c,12c) to produce 18:1-9c,12a.

alpina developing seeds, allowing the pVT-Crep1 plasmid to be constructed (derived from pVT100U) (Vernet *et al.,* 1987; Elble, 1992). Sequencing of *crep1* revealed a 375 amino acid protein possessing 59% amino acid sequence identity with the castor Δ12-hydroxylase and 56% identity with the *Arabidopsis* FAD2. The Crep1 gene was expressed in *Saccharomyces cerevisiae* (YN94-1 strain) and cultures were grown in the presence of 18:2–9c,12c, allowing for an accumulation of 18:1-9c,12a up to 0.3% of total fatty acid peaks detected during GC analysis. This work also identified and isolated an epoxygenase (Cpal1) from *Crepis palaestina* with genomic similarity to Crep1 (81% identity), but although sequence similarity was remarkable, no epoxy-fatty acids were detected in Crep1 cultures, nor was any acetylene-fatty acids detected in Cpal1 expressing cultures, leading researchers to suggest that small changes in amino acid sequences govern the different activities of these enzymes.

Crep1 contains the same 3 His-boxes as identified in other membrane-bound fatty acid desaturases. Other similarities shared with FAD2 include the requirement of NADH (or NADPH) as a cofactor, inhibition by cyanide, and that they are unaffected by carbon monoxide (a P450 inhibitor) and P450 antibodies (Lee *et al.,* 1998).

In addition to catalyzing the acetylenation of 18:2–9c,12c, Crep1 is also capable of introducing a *cis* or *trans* double bond at the Δ12 position of 18:1-9c, defining Crep1 as a bifunctional acetylenase/desaturase (Carlsson *et al.,* 2004). Crep1 was expressed in *S. cerevisiae* and shown to accumulate both 18:2-9c,12t and 18:2-9c,12c isomers to a 3:1 ratio as well as the acetylenase product, 18:1-9c,12a. Carlsson *et al.*, 2003, also determined that 18:2-9c,12c, and not 18:2-9c,12t was the precursor to 18:1-9c,12a. Kinetic isotope experiments have also been used to consider the mechanism of acetylenation by Crep1 which indicate that hydrogen abstraction initially occurs at C12 followed by a rapid hydrogen abstraction from C13 (Reed *et al.,* 2003). Crep1 reactions are shown in Figure 1.9.

Expression patterns of Crep1 were also studied in *C. alpina* and shown to be expressed the greatest in seed tissue where 18:1-9c,12a accumulates, but was also expressed in flower heads, although no 18:1-9c,12a accumulates there (Nam and Kappoch, 2007).

1.4 Structure/Function Relationships among Fatty Acid Desaturases and Variants

Although no crystal structures for FAD2s or their derivatives are available, studies considering their structure/function relationship have been conducted in which amino acid residues were exchanged and effects analyzed. This has led to the hypothesis that FAD2s and variants have two very important sites built within them which compose an active site and a substrate binding pocket (Shanklin and Whittle, 2003). This section considers some of the mutation analyses of fatty acid desaturases and their effect on chemoselectivity, substrate selectivity, and regioselectivity.

1.4.1 Mutation Analysis of Desaturases and their Homologs

Experiments involving the mutational analysis of desaturases were first conducted to discern the role of conserved amino acid motifs (His boxes). Mutation experiments which replaced alanine residues for the conserved histidine residues in rat Δ9-desaturase indicated their requirement for enzymatic activity (Shanklin *et al.,* 1994). Similar experiments have also indicated the importance of these motifs in acyl desaturases (Avelange-Macherel *et al.,* 1995), sterol desaturases (Taton *et al.,* 2000), and in AlkB hydroxylase (Shanklin and Whittle, 2003). As previously mentioned, these conserved amino acids are believed to coordinate a diiron cluster that interacts with molecular oxygen.

Mutational analysis has also been used to investigate the role of the cytochrome b_5 domain found in front-end desaturases, which identified an axial heme-binding residue critical for activity (Sayanova *et al.,* 1999). The N-terminal cytochrome b_5 domain from borage Δ6 desaturase has also been replaced with the b_5 domain from Arabidopsis Δ8 desaturase (Sayanova *et al.,* 2000). Yeast cultures harboring these chimaeras successfully accumulated 18:3-6c,9c,12c although in smaller amounts when compared to unmodified borage Δ6 desaturase expressed under similar conditions. In the same study, Sayanova *et al.*, 1999, also considered the importance of the conserved glutamine residue in Δ 5, Δ6, and Δ8 desaturases which replaces the conserved histidine residue within the third His-box of Δ12 and Δ15 desaturases. A mutant Δ6 desaturase which harbored histidine instead of glutamine did not produce 18:3-6c,9c,12c indicating the strict requirement of glutamine participation in front-end desaturases.

Variations in the amino acids involved around the active site and transmembrane domains are believed to affect the chemoselectivity and regioselectivity of FAD2 and their homologs (Broun *et al.,* 1998b). Chemoselectivity is the preferred chemical reaction for a substrate (e.g. hydroxylation vs. desaturation), whereas regioselectivity is the preferred location of bond making or breaking within a substrate. The substrate position within an enzyme is also an important determinant of an enzyme's functionality and selectivity, which is ultimately governed by specific amino acids which influence the binding of substrate molecules. In one related study (Broun *et al.,* 1998a), the functionality of a Δ12 desaturase was successfully converted to a Δ12 hydroxylase (m7FAD2) after exchanging as few as 4 amino acids. These amino acids are conserved within desaturase enzymes but differ from the conserved amino acids found in hydroxylases (LFAH2). The success in exchanging functionality between paralogs was largely due to their initial sequence alignments. After aligning hydroxylases (from the plants *Lesquerella fendleri* and *Ricinus communis*) and desaturases (from the plants *A. thaliana, Zea mays, Glycine max, R. communis, and Brassica napus*), the authors noted the non-conservative changes between both subsets of enzymes. The chemoselectivity was modified within enzymes after exchanging 7 conserved amino acids found in FAD2 but which were conserved but different in LFAH2. The ability to exchange the functionality between hydroxylases and desaturases as reported by Broun *et al.*, 1998a, suggested that acetylenase function could also be altered by using a similar approach.

Similar experiments have been conducted in which the chemoselectivity of an enzyme remained the same, but regioselectivity and substrate preference were altered. Cahoon *et al.*, 1997b, successfully altered a Δ6-palmitoyl-ACP desaturase into a Δ9-stearoyl-ACP desaturase by replacing 5 amino acids. These residues which affect acyl-chain length selectivity were shown by crystallography to line the substrate binding cavity. It is important to note that Cahoon's work was performed using soluble acyl-ACP desaturases and not FAD2s, for which no crystallographic data is available, and that although acyl-ACP desaturases and FAD2 are unrelated, both families are believed to have substrate binding cavities which may function similarly. The positional effects of a substrate binding cavity in desaturases and their role in determining regioselectivity are also discussed and modeled by Meesapyodsuk *et al.,* 2000b.

Two different desaturase genes from the fungus *Claviceps purpurea* have recently been examined, providing insight in the structural determinants of regioselectivity (Meesapyodsuk *et al.,* 2007). CpDes12 is a FAD2 enzyme which shares 87% identity with CpDesX, a FAD2 variant believed to have been derived from an ancestral gene duplication of CpDes12. CpDesX is a bifunctional desaturase capable of inserting a double bond at the Δ12 position, the Δ15 position, and at 'v+3' positions, which is the 3rd position from a preexisting double bond. During this study, reciprocal site-directed mutagenesis which exchanged amino acids between the CpDes homologs was used to show that two amino acids conferred the catalytic specificity of CpDesX and CpDes12. The two amino acids (valine/isoleucine at 152 and valine/alanine at 206) exist very close to the 3rd conserved His-box, being 3 amino acids upstream and 11 amino acids downstream (respectively). When amino acids at both these positions in CpDes12 were exchanged for those found in CpDesX, the FAD2 enzyme gained a 68-fold increase in the ratio of Δ15/Δ12 desaturation. Reciprocal experiments which exchanged CpDesX amino acid residues with those found in CpDes12 resulted in a dramatically reduced ratio of Δ15/Δ12 desaturation. The C-terminal sequences (residues 302 to 477) were also shown to be important contributors of catalytic specificity. Meesapyodsuk, 2007, comments that an enzyme's regioselective activity could possibly be determined by simply exchanging one amino acid.

1.5 Objectives

This work considers the structure/function relationship between FAD2s and acetylenases and provides insight into the structural determinants of their chemoselectivity and

regioselectivity. Conserved amino acids found in FAD2s were compared to those found in acetylenases and conserved amino acids which differed between each group were exchanged by site-directed mutagenesis in the acetylenase Crep1. More specifically, 38 FAD2 sequences were compared to 12 acetylenase sequences. Amino acid conservation amongst the desaturases and acetylenases were found to differ at 11 key positions. Amino acids at these positions within Crep1 were exchanged for the amino acids found in analogous positions within the FAD2 shared consensus sequence. Crep1 and Crep1 mutant enzymes were expressed in *S. cerevisiae* and cultured in either the presence or absence of 18:2–9c,12c, which acted as a substrate for Crep1 and mutant constructs. *S. cerevisiae* was chosen as a host since it does not possess endogenous FAD2 or acetylenase activity, but has been demonstrated to successfully express functional FAD2 and Crep1 when transformed with the appropriate vector (Covello and Reed, 1996; Lee *et al.,* 1998). Fatty acids produced by these transgenic yeast cultures were then analyzed by GC-FID and GC-MS. This study reports the effects that these point mutations had on fatty acid accumulation in transgenic yeast.

The objectives of this research were:
1- To collect and align FAD2 and acetylenase sequences,
2- To compare sequences and determine conserved amino acids which are found in either FAD2s or acetylenases, but not in both groups,
3- To design mutant *crep1* constructs which harbor designated point mutations determined from (2) (above),
4- To express *crep1* and mutants derived from *crep1* in INVSc1 yeast and to determine the effect(s) that point mutations had on the total fatty acid profiles in yeast.

Acetylenases have evolved more recently from FAD2 enzymes retaining much of the structural backbone which appear to be necessary for activity such as the conserved histidine residues believed to be involved in ion binding (Sperling *et al.,* 2003; Cahoon *et al.,* 2003). Only a small number of amino acid substitutions are involved in chemoselectivity. By exploring completely conserved amino acids which differ between acetylenases and desaturases, it is possible to elucidate which amino acids are the most important in determining chemoselectivity, stereoselectivity, and substrate selection.

2. MATERIALS AND METHODS

2.1 Determination of Amino Acid Targets

2.1.1 Sequence Selection and Alignments

Painstaking effort to avoid errors or omissions was given in the initial selection of definite FAD2 sequences since a large number of reported FAD2 are based on preliminary sequence data that lack investigation and definitive proof of function. Therefore the *Arabidopsis thaliana* FAD2 (ATHFAD2) (accession #NP_187819) was chosen to represent the core FAD2 sequence to use with the Basic Local Alignment Search Tool (BLASTP) (Altschul *et al.,* 1990) since it has been shown to be a specific FAD2 involved solely in the desaturation of 18:1-9c at the Δ12 position (Covello and Reed, 1996; Okuley *et al.,* 1994). BLASTP parameters were set to default. Sequences are listed in Table 3.1.

Acetylenase sequences were collected by conducting a BLASTP search using the *Petroselinum crispum* ELI12 sequence (PCRACET) (accession # AAB80697). PCRACET was used since it has been shown to be a true acetylenase (Kirsch *et al.,* 2000; Cahoon *et al.,* 2003). Twelve acetylenase sequences were collected in this manner (Table 3.1).

Amino acid sequences were aligned using CLUSTALW (Thompson *et al.,* 1994) with settings allowing the output alignment to be generated as they were originally input so that results clearly differentiated acetylenase and FAD2 sequences. All other parameters were set to their default setting. Acetylenase sequences were at the top of the input list (sequences 1-12) followed by the FAD2 sequences (sequences 13-50). CLUSTALW results were saved as alignment files (.aln) and later used as the input alignment files during further sequence comparative analysis (see below).

The *Dimorphotheca sinuata* acetylenase (DSIACET) and *Prunus armeniaca* desaturase (PRAFAD2) sequences (and others to a lesser extent) are truncated and were therefore excluded from alignments when considering enzyme family sequences upstream from the first conserved histidine box. This affects the conserved glycine/alanine amino acid residue found prior to the first His-box in desaturases/acetyleneases. DSIACET and PRAFAD2 truncated sequences were incorporated in all other sequence analyses.

2.1.2 Conservation of Amino Acids in Desaturases and Acetylenases

The Conserved Property Difference Locator software (CPDL) (Mayer *et al*., 2005) compares the conservation of amino acids between two groups of protein sequences and reports the amino acid differences that exist. The alignment files generated from CLUSTALW alignments were used as the input file for CPDL. CPDL analysis was conducted using strict conservation settings. During CPDL data entry, the *Top Group* entry was set from 1 to 12 (unless DSIACET was excluded in CLUSTALW alignments, in which case 1 to 11 was entered instead). This indicated that the top 12 entries in CLUSTALW were the 12 acetylenase sequences, followed by the remaining 38 FAD2 sequences. All other parameters were left at default except for *Conservation Level* which was tested for both *all* and *all or all but one*, allowing us to consider potential sequencing errors during the analysis.

2.2 Site-Directed Mutagenesis of pVTCrep1

2.2.1 Plasmid Preparation

The *crep1* gene was previously sub-cloned into the pVT100U vector (Vernet *et al.,* 1987; Lee *et al.,* 1998) (Figure 2.1) to create pVTCrep1 (not shown). These plasmids were used throughout this study as positive (pVTCrep1) and negative (pVT100U) controls (provided by D. Reed, NRC/PBI). The pVT100U and pVTCrep1 plasmids include the yeast autosomal replication factor (2μ ori), a uracil biosynthesis gene (URA3) for selection in yeast, an ampicillin resistance marker (ApR) for selection in *Escherichia coli*, an origin of replication for *E. coli* (f1 ori), and a constitutive alcohol dehydrogenase promoter (ADH3) for the continuous expression of cloned genes in yeast.

pVT100U and pVTCrep1 were transformed into *E. coli* TOP10F' (F'{*lac*I^Q Tn*10*(Tet^R)} *mcr*A (*mrr-hsd*RMD-*mcr*BC) θ80*lac*ZΔM15 Δ*lac*X74*rec*A1*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G) using a One Shot TOP10F' Competent Cell Kit (Invitrogen, Carlsbad, CA, USA) which uses heat shock on prepared competent cells. Transformed cells were selected for on Luria-Bertani broth (LB) + 100 μg/mL ampicillin grown overnight at 37°C. Protocol and reagent preparation was accomplished according to manufacturer's instruction (Invitrogen, Carlsbad, CA, USA).

Successful transformants were isolated by streaking single colonies onto new LB + 100 ug/mL ampicillin plates and culturing them overnight at 37°C. Single colonies from these

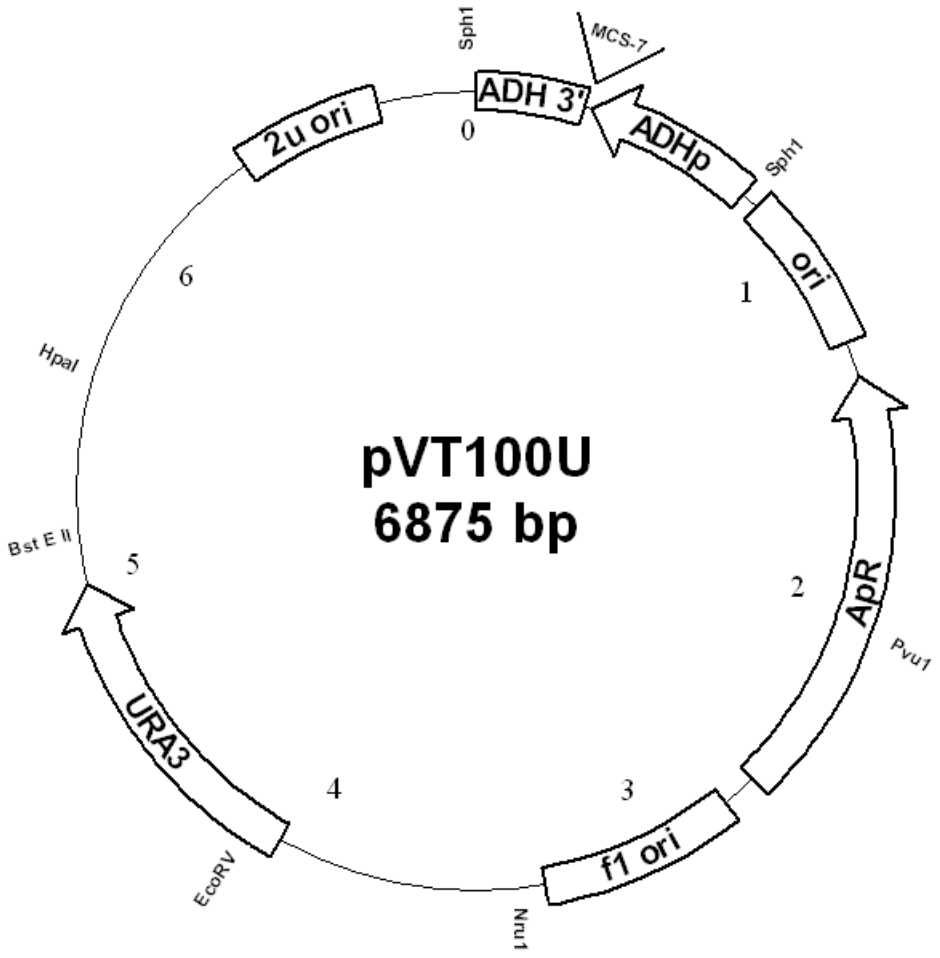

Figure 2.1 The pVT100U Plasmid
The pVT100U plasmid was used to transform INVSc1 yeast to create a negative control. This plasmid includes a multiple cloning site (MCS-7), the alcohol dehydrogenase promoter (ADH), ampicillin resistance marker for growth in *Escherichia coli*, a 2µ ori replication factor for autosomal replication in yeast, and the URA3 uracil biosynthesis gene. This plasmid is from Vernet *et al*., 1987.

isolated strains were then used to separately inoculate 10 mL of liquid LB + 100 µg/mL ampicillin and grown overnight at 37°C. Plasmids were purified using a Wizard *Plus* Midipreps DNA Purification System (Promega, Madison, WI, USA).

2.2.2 Site-Directed Mutagenesis

Point mutations at selected amino acid residues within pVTCrep1 were generated using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA, USA). This method allowed for simple point mutations in a four-step procedure in double-stranded DNA vector, eliminating the need for sub-cloning into M-13 bacteriophage vectors, single-stranded DNA rescue, use of restriction sites, or multiple transformations. The general method uses two oligonucleotide primers that include the desired mutation(s) and are complementary to the opposite dsDNA vector containing the gene of interest. It also uses a highly efficient *Pfu* Turbo DNA polymerase and, the dsDNA vector. After thermocycling, template DNA is treated with DpnI restriction enzyme, which cleaves only the methylated template DNA. This allows for selection of the newly generated unmethylated DNA which contains the point mutation.

SDM reactions used 2 µL of 6.05 ng/µL pVTCrep1 dsDNA template in combination with 5 µL of 10X supplied reaction buffer, 1.25 µL of each 0.100 µL/mL forward and reverse mutagenesis primers which were specific for the desired mutation (see Table 2.1), 1 µL of provided dNTP mixture, and 38.5 µL ddH_2O. Reaction mixtures were heated for 10 minutes at 95°C before 1 µL of *pfuUltra* HF DNA polymerase (2.5 U/µL) was added. Thermocycling was performed using either an MJ Research Thermal Cycler PTC-100 (BioRad, Hercules, CA, USA) or a Biorad I-Cycler. Thermocycling involved an initial 30s at 95°C followed by twelve cycles of the following: denaturation at 95°C for 30s; annealing at 55°C for 60s; elongation at 68°C for 8m 30s. Mutations N146H, F183L, and F183W required 16 cycles of thermocycling. Afterwards 1 µL of the provided *DpnI* restriction enzyme (10 U/µL) was added to reaction mixtures and allowed to digest for 1 hour at 37°C. All mutant constructs were validated by DNA sequencing (see below). Oligonucleotide primers used to introduce point mutations into pVTCrep1 were designed using PrimerX software (http://www.bioinformatics.org/primerx/documentation.html Copyright © 2003 by Carlo Lapid) (Table 2.1). Oligonucleotide primers were synthesized by DNA Technologies Group (Plant Biotechnology Institute, Saskatoon, Canada). dNTP mixture was obtained from Invitrogen (Carlsbad, CA, USA).

Table 2.1 Oligonucleotide Primers Used to Sequence and Mutagenize pVTCrep1
This table displays the name and sequence of oligonuceotide primers used to mutate or sequence the acetylenase Crep1. Each point mutation required a forward and reverse oligonucleotide primer (CF1/CR1. CF2/CR2, CF3/CR3) during site-directed mutagenesis.

Name	Usage	Sequence (5'→3')
CF1	Sequencing	GCAAGGTAGACAAGC
CR1	Sequencing	GTGGGTTTGAAAATGCCAGATGCA
CF2	Sequencing	CTGCACAATATTTCAAGC
CR2	Sequencing	AACCGGAACGAAAACCCCTACATT
CF3	Sequencing	AAGGTAGGGGGTCCAAGGGTGTAT
CR3	Sequencing	GTGGGAACCTGGGGGATGGAAAAA
F-G97A	Mutagenesis	CTTATGGGTCATCGCTCACGAATGCGGTC
R-G97A	Mutagenesis	GACCGCATTCGTGAGCGATGACCCATAAG
F-A139S	Mutagenesis	GCCACCGGAACCACCATTCTAACACAAATTCGCTTG
R-A139S	Mutagenesis	CAAGCGAATTTGTGTTAGAATGGTGGTTCCGGTGGC
F-N146H	Mutagenesis	CACAAATTCGCTTGACCACGATGAAGTTTACATC
R-N146H	Mutagenesis	GATGTAAACTTCATCGTGGTCAAGCGAATTTGTG
F-Y150F	Mutagenesis	CTTGACAACGATGAAGTTTTTATCCCCAAAAGCAAGGCC
R-Y150F	Mutagenesis	GGCCTTGCTTTTGGGGATAAAAACTTCATCGTTGTCAAG
F-F183L	Mutagenesis	CCTTCACCCTAGGCTTGCCTCTATACCTCTTTAC
R-F183L	Mutagenesis	GTAAAGAGGTATAGAGGCAAGCCTAGGGTGAAGG
F-L183W	Mutagenesis	CTTCACCCTAGGCTGGCCTCTATACCTC
R-L183W	Mutagenesis	GAGGTATAGAGGCCAGCCTAGGGTGAAG
F-K194R	Mutagenesis	CTCTTTACCAATATTTCCGGCAGAAAGTATGAAAGGTTTGCCAAC
R-K194R	Mutagenesis	GTTGGCAAACCTTTCATACTTTCTGCCGGAAATATTGGTAAAGAG
F-F259L	Mutagenesis	CAGTTTTAGGCGTGTTTATCTTTTTGGATATCATCACCTACTTGCACCAC
R-F259L	Mutagenesis	GTGGTGCAAGTAGGTGATGATATCCAAAAAGATAAACACGCCTAAAACTG
F-H266Q	Mutagenesis	CATCACCTACTTGCAACACACCCATCTGTC
R-H266Q	Mutagenesis	GACAGATGGGTGTGTTGCAAGTAGGTGATG
F-V304I	Mutagenesis	GTGTGCTCCATGATATTACACACACTCACG
R-V304I	Mutagenesis	CGTGAGTGTGTGTAATATCATGGAGCACAC
F-H306D	Mutagenesis	GTGTGCTCCATGATGTTACAGATACTCACGTTATGCATCATC
R-H306D	Mutagenesis	GATGATGCATAACGTGAGTATCTGTAACATCATGGAGCACAC
F-I317M	Mutagenesis	CATCTGTTTTCATACATGCCACACTATCATGCGAAG
R-I317M	Mutagenesis	CTTCGCATGATAGTGTGGCATGTATGAAAACAGATG

Some initial difficulty in generating F183W (where the conserved Crep1 phenylalanine residue at position 183 was converted to the conserved FAD2 tryptophan residue at the same position) led to the creation of F183L. This facilitated the point mutation to tryptophan at the 183rd position since L183W was easily generated afterward. After F183L was created, it was retained and used in subsequent experiments. All point mutations were successfully generated within pVTCrep1 resulting in 12 mutant plasmids.

2.2.3 Sequencing

All DNA sequencing was performed using an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with a 3730x1 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) (DNA Sequencing Lab, Plant Biotechnology Institute, Saskatoon, Saskatchewan). Three separate primer pairs shown in Table 2.1 were used to sequence plasmids; CF1/CR1, CF2/CR2, CF3/CR3 include both the forward and reverse primers.

2.2.4 Transformation of InvSc1 Yeast Cultures

All plasmid constructs were individually transformed into *S. cerevisiae* strain InvSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*) using a *Saccharomyces cerevisiae* EasyComp Transformation Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Yeast cells were made competent by the lithium acetate method (Ito *et al.,* 1984; Gietz and Schiest, 1991), using reagents that were provided in the kit. Untransformed competent cells were cultured in yeast extract peptone dextrose (YPD) media prepared according to manufacturer's instructions. Transformants were selected on minimal synthetic dextrose media supplemented with amino acids excluding uracil (SD-URA) (Bio101 Inc., Vista, CA, USA). Single colonies were restreaked onto new SD-URA plates and cultured overnight at 37°C.

2.3 Analysis of InvSc1 Yeast Harboring pCrep1 and Mutant Constructs

2.3.1 Growth Conditions

Starter cultures of transformed yeast were grown for 48 hours at 28°C in 1 mL SD-URA broth. 100 µL of starter culture was then used to inoculate 10 mL SD-URA in 50 mL Erlenmeyer flasks. Linoleic acid (18:2-9c,12c) (Nu-Chek Prep Inc., Elysian, MN, USA) was also added to cultures (100 µg/mL free acid in 0.1% tergitol v/v) which were to be analyzed for 18:1-9c,12a and 18:2-9c,12c and 18:2-9c,12t isomers. All experiments were performed in triplicate cultures on three different occasions. Cultures were grown for three days at 20°C followed by another three days at 15°C on a platform shaker (200 rpm) to increase fatty acid product, as described by Reed *et al.*, 2003.

2.3.2 Sample Preparation for Gas Chromatography and Mass Spectrometry Analysis

Cultures grown in the presence of 18:2-9c,12c were centrifuged (1500 G for 5 min), washed (1 mL 10% tergitol v/v), centrifuged at 1500 G for 5 min, and rinsed twice using 5 mL of ddH_2O to remove excess tergitol and18:2-9c,12c. Cultures grown in the absence of 18:2-9c,12c were centrifuged (1500 G for 5 min), rinsed (5 mL ddH_2O), and centrifuged. 100 µL of 17:0 (100 µg/mL in methanol) (Nu-Chek Prep Inc., Elysian, MN, USA) was added to each sample to act as an internal standard. Sample preparation involved the saponification of fatty acids using 1 mL of 10% KOH (w/v) in methanol and heating the reaction mixture in a sealed glass tube with a Teflon-lined lid to 80°C for exactly one hour. The heating of extracts was performed behind an acrylic glass shield as a safety precaution. Mixtures were then cleared of sterols by extracting with 1 mL hexane, followed by acidification of free fatty conjugate bases using 500 µL of 10% acetic acid. Fatty acids were then extracted using 2 mL hexane and later concentrated under a nitrogen stream. The residue was methylated by heating to 60°C in the presence of 1 mL 1% sulfuric acid (v/v) in dry methanol for one hour. Fatty acid methyl esters (FAMEs) were extracted from the mixture using 2 mL hexane after addition of 1 mL ddH_2O. The FAMEs were then dried under a N_2 stream to dryness followed by the addition of 200 µL ethyl acetate. This method is adapted from that of Reed *et al.*, 2003.

2.3.3 Fatty Acid Analysis by Gas Chromatography and Mass Spectrometry

The hexane extracts from the methylation reactions were analyzed by GC-MS using an Agilent 6890N GC with a DB-23 column (30 m x 0.25 mm i.d., J&W Scientific), 25:1 split flow injector at 225°C, and a column oven temperature program of 160°C for 1 min then 4°C/min to 240°C connected to an Agilent 5973N mass selective detector in electron impact mode under standard conditions (70 eV). GC analysis was done using an Agilent 6890 GC with a similar column and the oven under the same conditions connected to a flame ionization detector. Quantitations for both detection systems were done using Chemstation software (Agilent) calculated peak area integrations and comparing areas to 17:0 added to samples as an internal standard and applying appropriate response factors for the necessary comparisons. For FID analysis, response factors of the fatty acid methyl esters being quantitated were determined to be 1.0 on a weight basis. This method is adapted from that of Reed *et al.*, 2003.

3. RESULTS

3.1 Sequence Analysis of Fatty Acid Desaturases and Acetylenases

To explore the structure/function relationship between acetylenases and desaturases, their primary structures were considered. Amino acid conservation within but not between each group of enzymes was analyzed using a series of bioinformatics tools. Several sequences for FAD2s with reported desaturase activity were collected from Genbank. A sequence similarity search using ATHFAD2 was also conducted using BLASTP from which several FAD2 sequences were obtained. Results were pooled and used to create a database composed of 38 FAD2 sequences. Twelve acetylenase sequences were also collected using similar methods. All but one of the retrieved acetylenase sequences have been reported by the same source (Cahoon *et al.,* 2003). These 12 acetylenase sequences were also added to our database. The 38 FAD2 sequences that were chosen represent 17 plant families and one algae family (Chlorellaceae). In contrast, the 12 acetylenase sequences are dispersed in only 3 plant families: Asteraceae, Apiaceae, and Araliaceae. Plants from these families include sunflower, strawflower, Black-eyed Susan, parsley, fennel, carrot, African daisies, marigold, and English ivy (Table 3.1)

All sequences were then aligned. Although some acetylenase sequences were truncated at the N-terminus as opposed to complete FAD2 sequences (*i.e.* FVUACET, DCAACET, HBRACET, DSIACET, RHIACET, COFACET), they were included in the overall analysis (Appendix A). The analysis was conducted with and without these sequences and results indicated that amino acid residue conservation in the truncated N-terminal positions did not greatly influence the overall final results; that is, no conservation of amino acids amongst acetylenases or FAD2s differ within their N-terminal other than the glycine residue at position 97 within Crep1.

Aligned sequences were then compared using the CPDL software. The CPDL software allowed for the determination of amino acids which are conserved in either the FAD2 or acetylenase enzymes, but that differ in the other group of enzymes. Results highlighted 11 amino acid residues that are conserved in either one group of enzymes but are different in the other group of enzymes (Figure 3.1).

Table 3.1 Abbreviations of Sequences Used to Determine Conservation in FAD2s and Acetylenases

Δ12 fatty acid desaturases and acetylenase sequences that were used in this study are divided into two groups (indicated by the label along the left margin). Names and abbreviations of the sequences were designated based on genus, species, and function. The strongest evidence of the designated activity is listed under the evidence column.

	Name	Family	Genus Species	NCBI Accession #	Evidence
ACETYLENASES	PCRACET (ELI12)	Apiaceae	*Petroselinum crispum*	AAB80697.1	transgenic soybean expression
	PCRACET-2	Apiaceae	*Petroselinum crispum*	AAG23923.1	89% AA ID to PCRACET (ELI12)
	PCRACET-3	Apiaceae	*Petroselinum crispum*	AAG23924.1	91% AA ID to PCRACET (ELI12)
	FVUACET	Apiaceae	*Foeniculum vulgare*	AAO38034.1	91% AA ID to PCRACET (ELI12)
	DCAACET	Apiaceae	*Daucus carota*	AAO38033.1	91% AA ID to PCRACET (ELI12)
	HANACET	Asteraceae	*Helianthus annuus*	CAA76158.2	transgenic soybean expression
	RHIACET	Asteraceae	*Rudbeckia hirta*	CAB64256.1	94% AA ID to HANACET
	HBRACET	Asteraceae	*Helichrysum bracteatum*	AAO38037.1	89% AA ID to HANACET
	DSIACET	Asteraceae	*Dimorphotheca sinuata*	AAO38036.1	89% AA ID to HANACET
	COFACET	Asteraceae	*Calendula officinalis*	AAO38035.1	transgenic soybean expression
	CALACET	Asteraceae	*Crepis alpina*	AAO38032.1	transgenic yeast expression
	HHEACET	Araliaceae	*Hedera helix*	AAO38031.1	transgenic soybean expression
Δ12 DESATURASES	ATHFAD2	Brassicaceae	*Arabidopsis thaliana*	NP_187819.1	transgenic yeast expression
	BCAFAD2	Brassicaceae	*Brassica carinata*	AAK26633.1	91% AA ID to ATHFAD2
	BJUFAD2	Brassicaceae	*Brassica juncea*	CAA62578.1	90% AA ID to ATHFAD2
	BRAFAD2-1	Brassicaceae	*Brassica rapa*	CAG26981.1	90% AA ID to ATHFAD2
	BNAFAD2	Brassicaceae	*Brassica napus*	AAF78778.1	90% AA ID to ATHFAD2

Δ12 DESATURASES	VGAFAD2	Asteraceae	*Vernonia galamensis*	AAF04094.1	transgenic yeast expression
	CPAFAD2	Asteraceae	*Crepis palaestina*	CAA76157	73% AA ID to ATHFAD2
	HANFAD2	Asteraceae	*Helianthus annuus*	AAL68983.1	transgenic yeast expression
	COFFAD2	Asteraceae	*Calendula officinalis*	AAK26633.1	transgenic yeast expression
	GHIFAD2-1	Malvaceae	*Gossypium hirsutum*	AAQ16653.1	76% AA ID to ATHFAD2
	GHIFAD2-2	Malvaceae	*Gossypium hirsutum*	AAQ16654.1	76% AA ID to ATHFAD2
	GHIFAD2-3	Malvaceae	*Gossypium hirsutum*	AAL37484.1	76% AA ID to ATHFAD2
	SOLFAD2	Chenopodiaceae	*Spinacia oleracea*	BAC22091.1	transgenic porcine expression
	TMAFAD2	Tropaeolaceae	*Tropaeolum majus*	AAV52834.1	77% AA ID to ATHFAD2
	CPEFAD2	Cucurbitaceae	*Cucurbita pepo*	AAS19533.1	78% AA ID to ATHFAD2
	TKIFAD2	Cucurbitaceae	*Trichosanthes kirilowii*	AAO37752.1	transgenic yeast expression
	JCUFAD2	Euphorbiaceae	*Jatropha curcas*	ABA41034.1	77% AA ID to ATHFAD2
	BOFFAD2	Boraginaceae	*Borago officinalis*	AAC31698.1	77% AA ID to ATHFAD2
	SINFAD2	Pedaliaceae	*Sesamum indicum*	AAX11454.1	75% AA ID to ATHFAD2
	LUSFAD2	Linaceae	*Linum usitatissimum*	ABB05230.1	75% AA ID to ATHFAD2
	SCOFAD2	Solanaceae	*Solanum commersonii*	CAA63432.1	74% AA ID to ATHFAD2
	NTAFAD2	Solanaceae	*Nicotiana tabacum*	AAT72296.2	60% AA ID to ATHFAD2
	ZMAFAD2	Poaceae	*Zea mays*	BAE93382.1	69% AA ID to ATHFAD2
	OSAFAD2	Poaceae	*Oryza sativa*	XP_467474.1	67.5% AA ID to ATHFAD2
	PAMFAD2	Lauraceae	*Persea americana*	AAL23676.1	59% AA ID to ATHFAD2
	HBRFAD2	Euphorbiaceae	*Hevea brasiliensis*	AAY87459.1	transgenic yeast expression
	VFOFAD2	Euphorbiaceae	*Vernicia fordii*	AAN87573.1	transgenic yeast expression

Δ12 DESATURASES	OEUFAD2-1	Lamiales	*Olea europaea*	AAW63040.1	transgenic yeast expression
	OEUFAD2-2	Lamiales	*Olea europaea*	AAW63041.1	transgenic yeast expression
	ADUFAD2	Fabaceae	*Arachis duranensis*	AAF82294.1	transgenic yeast expression
	AHYFAD2-1	Fabaceae	*Arachis hypogaea*	AAY53559.1	99.2% AA ID to ADUFAD2
	AHYFAD2-2	Fabaceae	*Arachis hypogaea*	AAB84262.1	70.5% AA ID to ATHFAD2
	AHYFAD2-3	Fabaceae	*Arachis hypogaea*	AAK67829.1	70.5% AA ID to ATHFAD2
	AMOFAD2	Fabaceae	*Arachis monticola*	AAX14399.1	98.9% AA ID to ADUFAD2
	AIPFAD2	Fabaceae	*Arachis ipaensis*	AAF82295.1	transgenic yeast expression
	GMAFAD2-1	Fabaceae	*Glycine max*	BAD89862.1	76% AA ID to ATHFAD2
	GMAFAD2-2	Fabaceae	*Glycine max*	P48631	76% AA ID to ATHFAD2
	CVUFAD2	Chlorellaceae	*Chlorella vulgaris*	BAB78716.1	transgenic yeast expression

This study determines which amino acid residues within acetylenases and desaturases play a role in differentiating the different activities either assumed or observed. Based on CPDL, 11 amino acids were targeted within the Crep1 protein for site-directed mutagenesis. Amino acids at these locations within Crep1 were singularly exchanged for residues conserved at the same location in FAD2s. An example can be seen in Figure 3.1 where at position 336 within the CPDL results (highlighted by a red arrow) indicate an isoleucine residue (I) which is always conserved within acetylenases, whereas methionine (M) is always conserved within FAD2s. The isoleucine residue within the Crep1 sequence was mutated to a methionine residue. The identity and locations of all amino acids targeted for site-directed mutagenesis, as well as the names of the plasmids constructed, are summarized in Table 3.2. In addition to the 11 plasmids generated, another plasmid (pSG005) was also designed to explore the effects of replacing a conserved Crep1 residue with a residue that is not found in either FAD2s or acetylenases (F183L). Thus two changes were conducted at amino acid residue 183 within Crep1 (phenylalanine) so that one change introduced the conserved FAD2 residue (tryptophan), whereas the other introduced an amino acid residue not found in either acetylenases or FAD2s

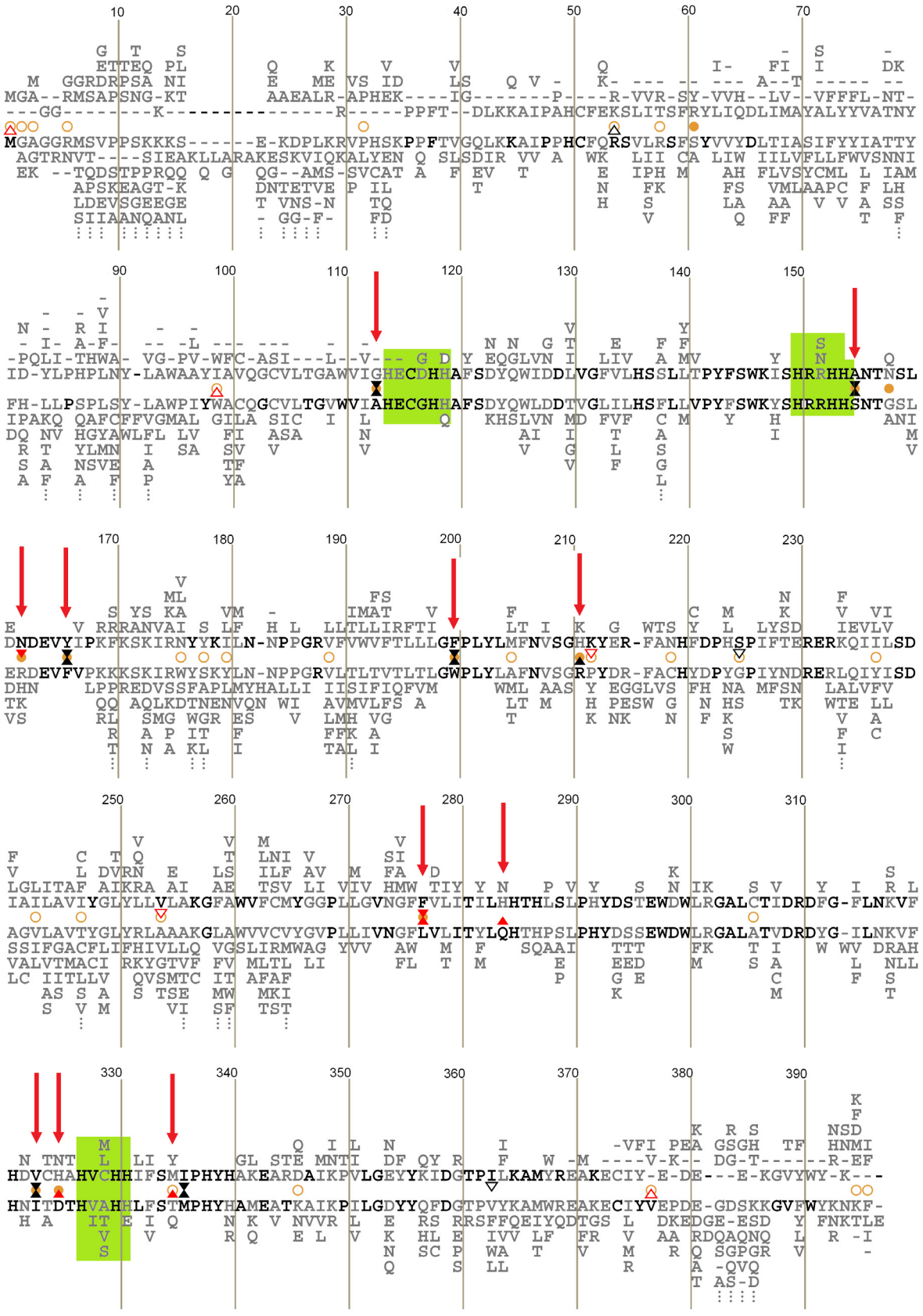

Figure 3.1 Determination of Amino Acid Conservation Within Acetylenases and FAD2s by the Use of the Conserved Property Difference Locator

Abridged Conserved Property Difference Locator (CPDL) results considering 12 acetylenase protein sequences compared to 38 Δ12 fatty acid desaturase (FAD2) sequences. The amino acids above the midline represent the consensus sequence of the 12 acetylenases whereas the amino acids below the midline occur in FAD2 enzymes. Residues beyond these positions (up for acetylenases and down for desaturases) are positioned farther from the midline as their frequency of occurrence within the respective group of enzymes diminishes. Residues in bold represent conserved amino acids. Color-filled triangles indicate conserved amino acids within either group of enzymes that is not found in the other group. Circles indicate conservation of properties of amino acids which differ between acetylenases and FAD2s. Symbols not filled with color indicate that the conserved amino acid or property from one group of enzymes is found in at least one sequence in the other group of enzymes. Eleven candidate amino acid targets for site-directed mutagenesis are indicated by red arrows. These amino acids are conserved in either acetylenases or FAD2 enzymes. Three conserved histidine motifs found in all acetylenase and desaturase sequences are highlighted in green. Note that at position 113, conservation of glycine within acetylenases is not denoted by CPDL due to the inclusion of N-terminal truncated sequences.

at this position (leucine). The CPDL results indicate other sites of interest where although no specific conservation exists for a single amino acid, amino acid properties are conserved in at least one group. These other sites were not considered any further in this study. In an attempt to visualize the location of the targeted amino acids, the topology of Crep1 was explored. A pictorial representation of Crep1 was produced by using a combination of software. Transmembrane spanning domains were predicted using the TMHMMv2.0 software suite (Krogh *et al*., 2001) (Figure 3.2A). A linear model of Crep1 highlighting the predicted transmembrane locations, the conserved His boxes, and the targeted amino acid positions was then created using DirectDomain software (Fink and Hamilton, 2007) (Figure 3.2B). In congruence with similar topological representations of desaturases previously reported (Stuckey *et al.*, 1989; Shanklin *et al.,* 1994; Minto *et al.,* 2002), these results were used to generate a two-dimensional representation of Crep1 which highlights amino acids targeted for site-directed mutagenesis (Figure 3.3). Results indicate that 7 of the 11 conserved amino acids which differ between FAD2 and acetylenase enzymes exist in close proximity (within 12 amino acids) to the conserved His boxes. The remaining 4 targeted amino acids within Crep1 (F183, K194, H266, and F259) exist farther from the conserved His boxes. F183 appears to exist

Table 3.2 Conserved Amino Acids in FAD2s and Acetylenases

This table displays the amino acid conservation in two groups of enzymes; acetylenases and Δ12 fatty acid desaturases (FAD2s). Positions of targeted amino acids in Crep1 are listed in which the corresponding amino acid is completely conserved in at least one group of enzymes but differ from homologous amino acids found in the other group of enzymes. Amino acids residues in Crep1 were singularly replaced with residues found at the same location in the FAD2 consensus sequence.

Plasmid Name	**Position of residue within Crep1**	**Crep1 Residue**	**FAD2 Residue**
SG001	97	Glycine (G)	Alanine (A)
SG002	139	Alanine (A)	Serine (S)
SG003	146	Asparagine (N)	Histidine (H)
SG004	150	Tyrosine (Y)	Phenylalanine (F)
SG005	183	Phenylalanine (F)	Leucine (L)
SG006	183	Phenylalanine (F)	Tryptophan (W)
SG007	194	Lysine (K)	Arginine (R)
SG008	259	Phenylalanine (F)	Lysine (L)
SG009	266	Histidine (H)	Glutamine (Q)
SG010	304	Valine (V)	Isoleucine (I)
SG011	306	Histidine (H)	Aspartic Acid (D)
SG012	317	Isoleucine (I)	Methionine (M)

within the 3rd transmembrane domain; K194 appears to exist within the lumen of the endoplasmic reticulum following the 3rd transmembrane domain. Lastly F259 and H266 appear near the putative cytosolic end of the 4th transmembrane domain (Figure 3.3).

3.2 The Detection of Fatty Acids by Gas Chromatography

This results section deals with the detection and identification of fatty acids from transgenic yeast cultures. The quantitation of fatty acids is presented in the later sections that follow. Initially, the fatty acids produced by the negative control (*i.e.* yeast harboring pVT100U) were analyzed when grown in the presence or absence of 18:2–9c,12c. The positive control (yeast harboring pVTCrep1), which expresses the unmodified form of Crep1, was investigated, followed by the analysis of the mutant constructs. Throughout the experiments,

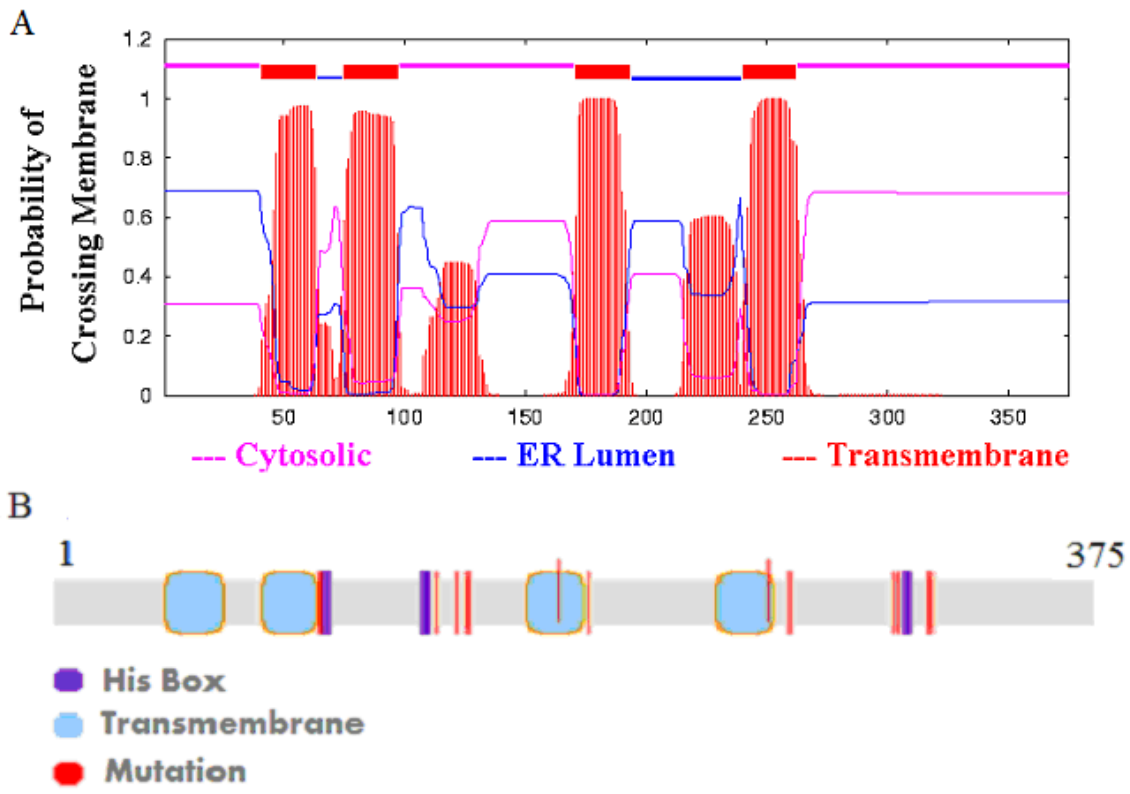

Figure 3.2 Hydrophobicity of Crep1 used to Determine Transmembrane Domains
(A) The predicted transmembrane domains for the acetylenase Crep1 determined by TMHMMv2.0 (Krogh *et al.,* 2001). The TMHMM software predicts the location of 4 transmembrane spanning domains within Crep1. The predicted location of Crep1 domains are indicated above the plot by a colored line, where the pink lines indicate the cytosol, the blue lines represent the lumen of the endoplasmic reticulum, and the red lines indicate positioning within the ER membrane.
(B) A one-dimensional schematic of Crep1 acetylenase created using DomainDraw (Fink and Hamilton, 2007). This schematic highlights predicted transmembrane spanning domains (blue), conserved His boxes within Crep1 (purple), and the location of point mutations introduced within Crep1 (red). This data was used to generate a 2-dimensional topology of Crep1 protein presented in Figure 3.3.

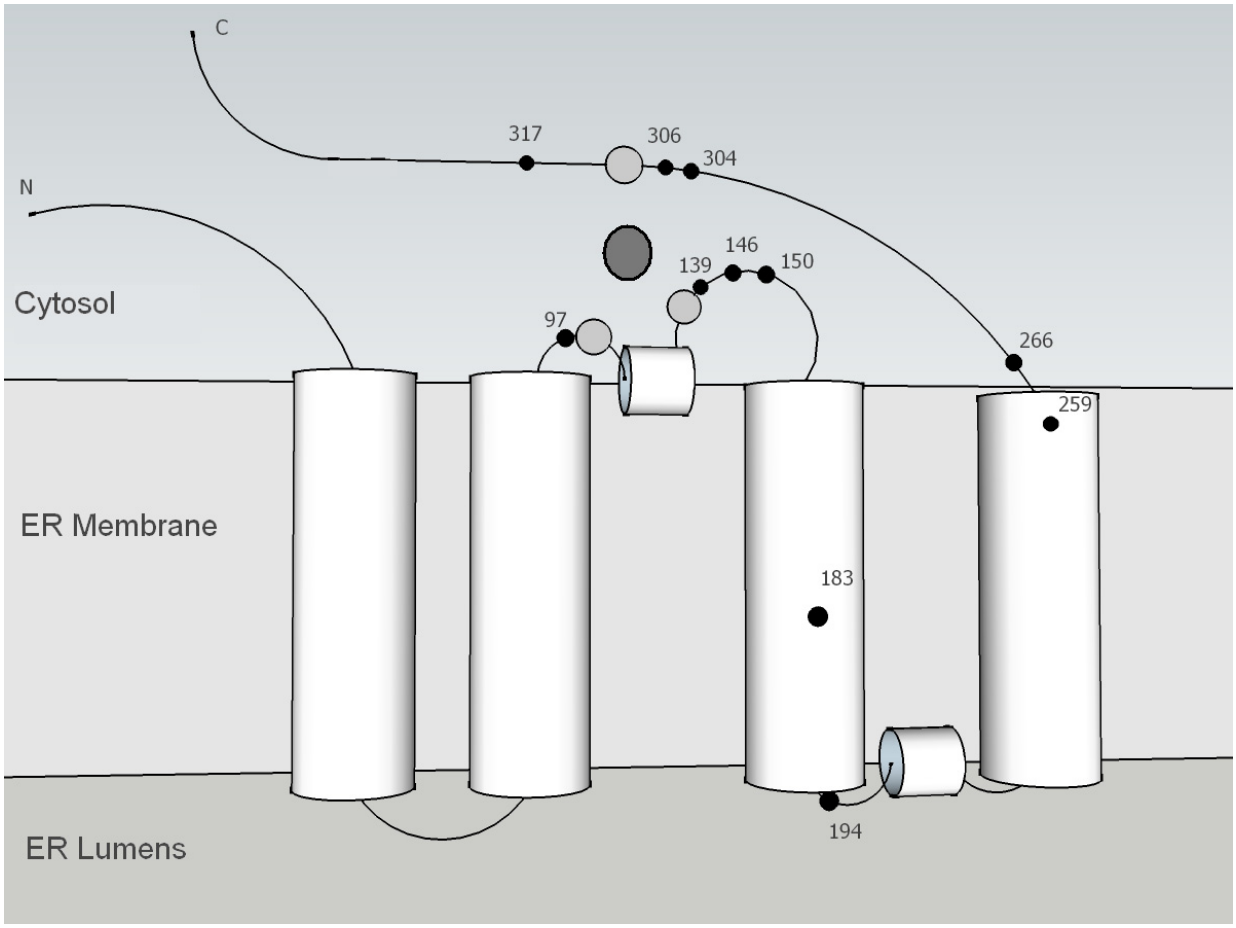

Figure 3.3 The Predicted Topology of Crep1 Indicative of Conserved Amino Acids which are Targets for Site-Directed Mutagenesis
A two-dimensional schematic of the acetylenase Crep1 composed of 375 amino acids. This diagram indicates the locations of selected amino acids targeted for site-directed mutagenesis within the Crep1 protein (black dots). Other important structures are highlighted: 4 predicted transmembrane domains (cylinders traversing ER membrane), conserved His boxes (○)believed to coordinate a di-iron cluster (●) at the active site, where hypothetical bonds are represented by dotted lines.

fatty acids that make up the majority of all the total fatty acids produced in cultures were considered including hexadecanoic acid (16:0), *cis*-9-hexadecenoic acid (16:1–9c), octadecanoic acid (18:0), and *cis*-9-octadecenoic acid (18:1–9c). In addition to the major fatty acids detected, the minor amounts of 16:2-9c,12c and 16:2-9c,12t isomers, 18:2-9c,12c and 18:2-9c,12t isomers, and 18:1-9c,12a were also examined. These fatty acids constitute the total fatty acid profile used throughout the following experiments. In addition, heptadecanoic acid

(17:0) was added during sample preparation as an internal standard, which was detected throughout all the experiments but was not included as part of the total fatty acid content.

INVSc1 yeast was transformed with empty plasmid (pVT100U) to generate a negative control and then cultured for 6 days. Fatty acid accumulation in this strain was analyzed by GC-FID (Figure 3.4A). Four fatty acids were found to constitute the majority (>90%) of total fatty acids (TFA) detected: 16:0, 16:1–9c, 18:0, and 18:1–9c. Trace amounts of 18:2-9c,12c were also detected. Other fatty acids, such as dodecanoic acid (12:0) and tetradecanoic acid (14:0) were also produced, but always in minute quantities (<1% TFA) and were therefore not included in the total fatty acid profiles of cultures during this study.

Negative controls were also grown for 6 days in the presence of 18:2-9c,12c followed by fatty acid analysis by GC-FID. In Figure 3.4B, the same 4 simple fatty acids were detected as in the previous experiment, as well as the (added) fifth fatty acid, 18:2-9c,12c. Additionally trace amounts of 16:2-9c,12t were also detected.

When INVSc1 yeast was transformed with pVTCrep1 and grown for 6 days, GC-FID analysis revealed the same 4 endogenous fatty acids as detected in negative controls (*i.e.* 16:0, 16:1–9c, 18:0, 18:1–9c) (Figure 3.5A), as well as 16:2-9c,12t, 18:2-9c,12t and 18:2-9c,12c (Figure 3.6A).

Similar experiments using INVSc1 cultures bearing mutations F259L and V304I within Crep1 revealed another 16:2 isomer not detected in other cultures; 16:2-9c,12c (Figure 3.6B; results for V304I not shown).

Yeast strains expressing Crep1 were also grown for 6 days in the presence of 18:2-9c,12c followed by fatty acid analysis by GC-FID. The same endogenous fatty acids detected in negative controls were present. Other fatty acids present in these cultures were 16:2-9c,12t and 18:1-9c,12a. Although the presence of 16:2 isomers could be detected, 18:2 isomers could not be resolved due to the addition of 18:2-9c,12c (Figures 3.5B and 3.7).

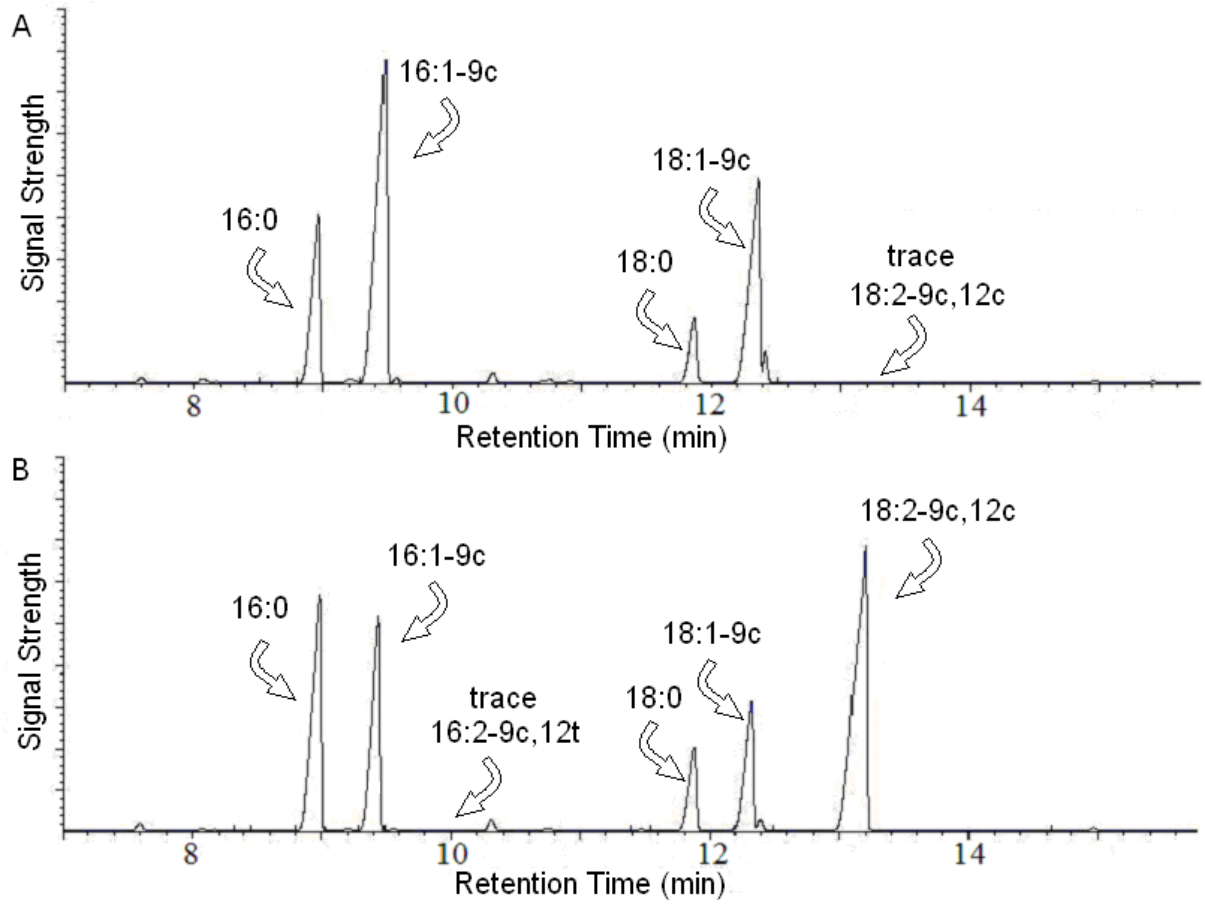

Figure 3.4 Gas Chromatography of Fatty Acids in Yeast Negative Controls
GC-FID traces are shown for the INVSc1 yeast strain transformed with pVT100U (empty vector) after growth for 6 days in the absence (A) and presence (B) of 18:2 9c,12c.

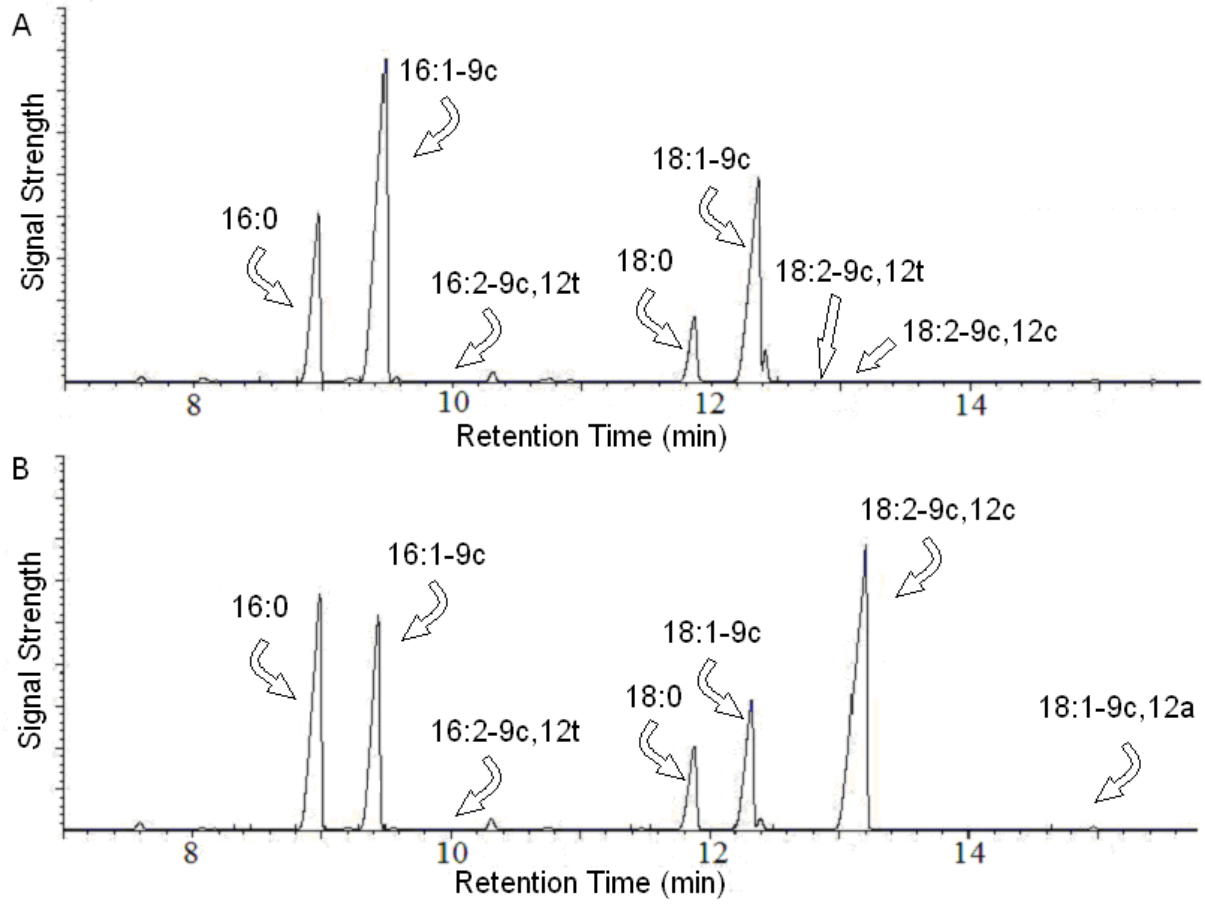

Figure 3.5 Gas Chromatography of Fatty Acids from Yeast Expressing Crep1
GC-FID traces are shown for the INVSc1 yeast strain expressing the acetylenase Crep1 following 6 days of growth in the absence (A) or presence (B) of 18:2-9c,12c.

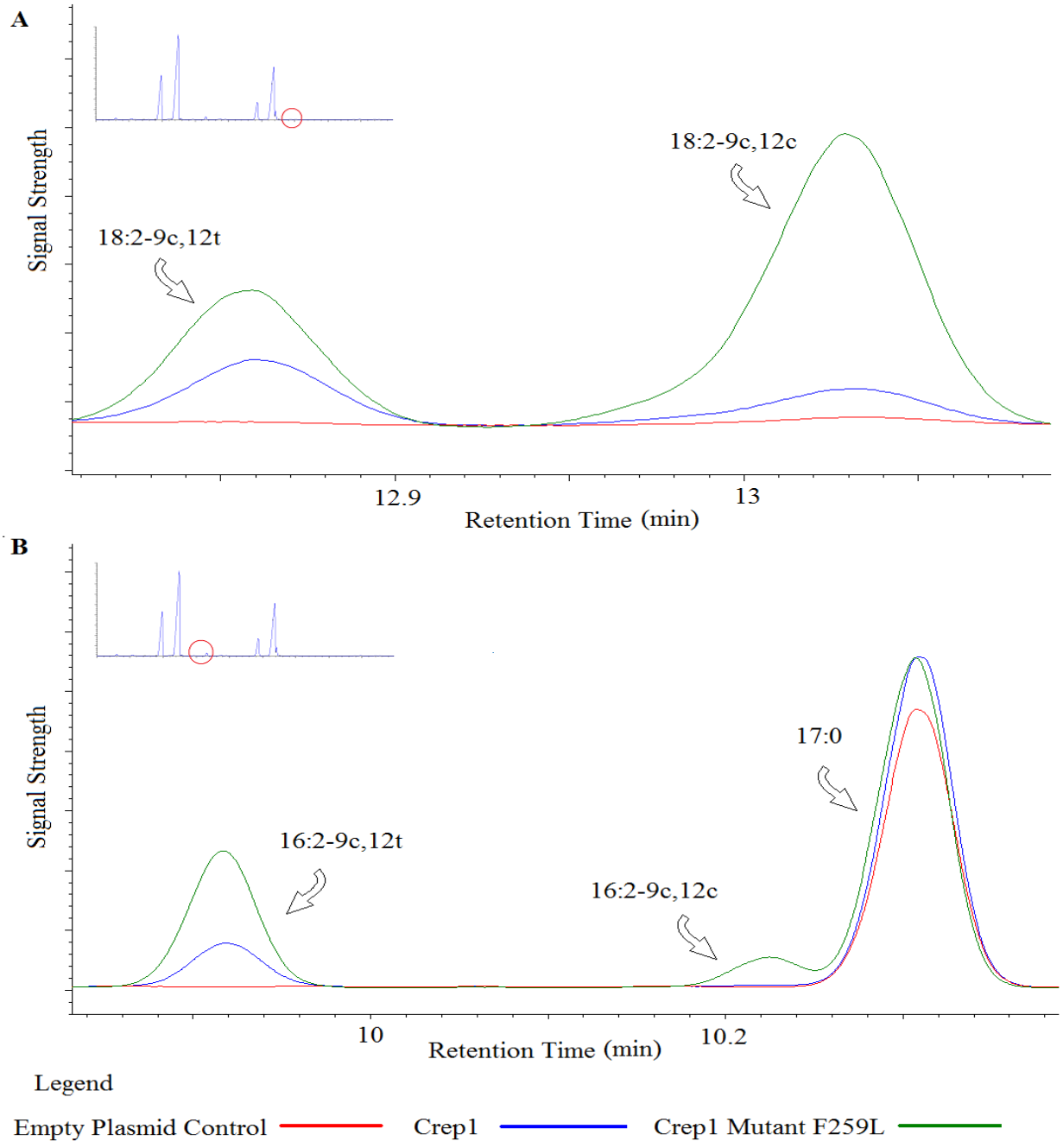

Figure 3.6 The Accumulation of Geometric Isomers in Yeast Cultured in the Absence of 18:2-9c,12c

An inset of the 18:2 isomer region (A) and 16:2 isomer region (B) from Figure 3.5A (also seen as an insert in the top left corner of panel (A) and (B) indicates the elution order for geometric isomer of 18:2 in INVSc1 yeast cultures expressing Crep1 (blue), harboring pSG008 (F259L) (green), and negative controls harboring empty plasmid pVT100U (red).

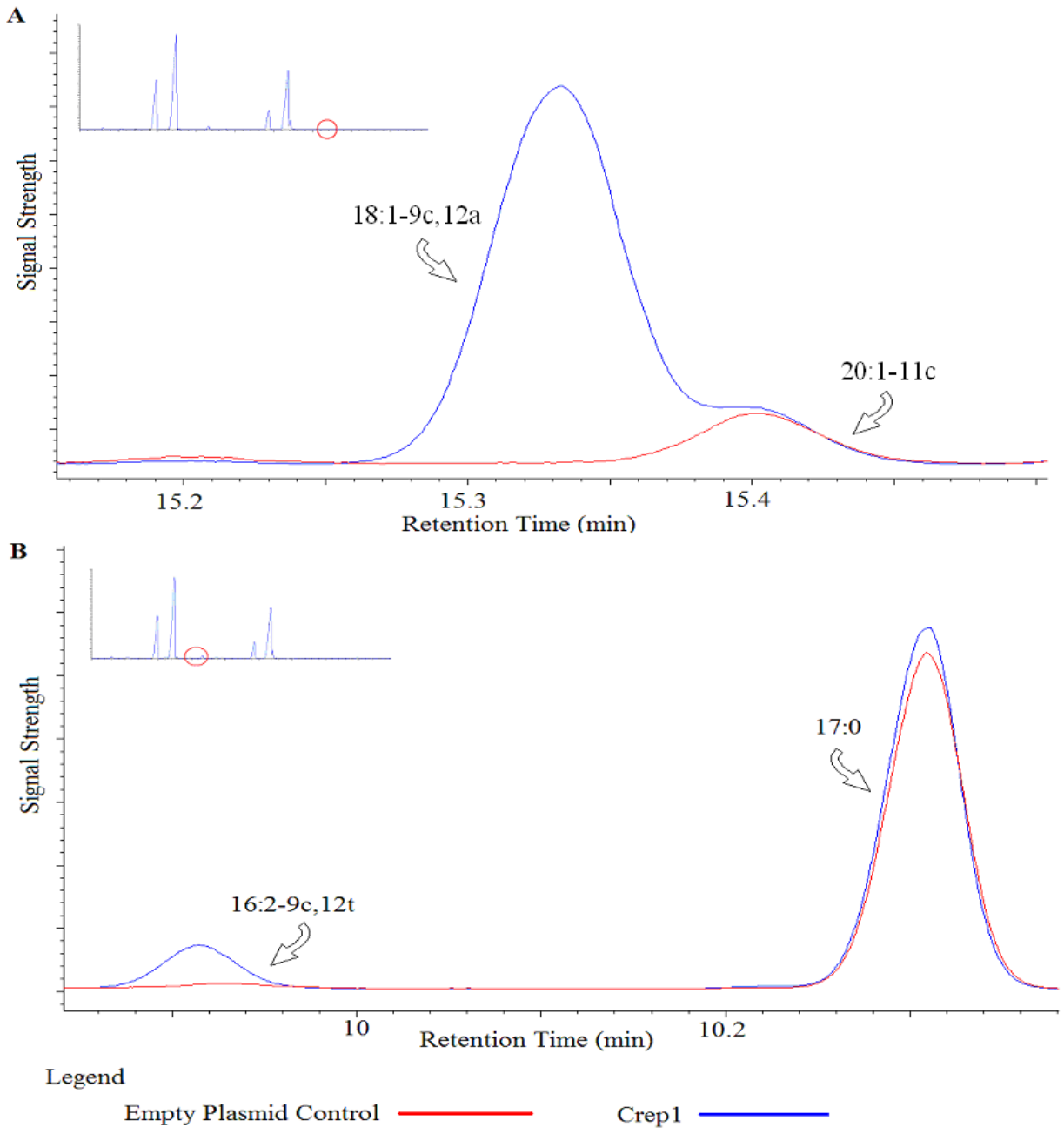

Figure 3.7 GC Identification of 18:1-9c,12a and 16:2-9c,12t Accumulating in Yeast Cultured in the Presence of 18:2-9c,12c

An inset of the 18:1-9c,12a region (A) and the 16:2 isomer region (B) from Figure 3.5B displays the accumulation of 18:1-9c,12a (A) and 16:2 isomers (B) when INVSc1 yeast was transformed with either pVTCrep1 (blue) or empty plasmid (red) and grown for 6 days in the presence of 18:2-9c,12c.

3.2.1 Identification of Fatty Acids

Fatty acids were identified by either the use of purified standards using GC-FID analysis, GC-MS, or by both methods. Simple fatty acids such as 16:0, 16:1–9c, 18:0, and 18:1–9c were easily identified by their retention times and confirmed by the use of standards during GC-FID analysis.

18:2 isomers were identified by using a mixture of known standards during GC analysis. The standard included all geometric isomers of 18:2Δ9,12. This allowed for the identification of 18:2-9c,12c and 18:2-9c,12t, as well as allowing us to discern their order of elution. 18:2-9c,12t eluted at the 12.8 min mark followed closely by 18:2-9c,12c which eluted at 13 min (Figure 3.6A). The identification of the 18:2 isomers was also confirmed by the use of GC-MS.

The identities of 16:2 isomers were not known during early GC-FID analysis. The appearance of peaks in yeast cultures expressing Crep1 or Crep1 mutant constructs required identification (Figure 3.6B). No purified 16:2 standards were available for the identification of 16:2 isomers by GC-FID, hence chemical composition was identified by mass spectrometry whereas stereochemistry was assigned based on the elution pattern of 18:2 isomers. The peak appearing at the 9.9 min mark (16:2-9c,12t) in cultures expressing Crep1 or Crep1 mutants (and in trace amounts in negative cultures grown in the presence of 18:2-9c,12c), as well as the peak appearing at the 10.2 min mark (16:2-9c,12c) in yeast harboring plasmids pSG008 (F259L) or pSG010 (V304I) were analyzed by GC-MS (Figure 3.8). The fragmentation pattern obtained from the respective peaks were compared to our chemical database and identified as 16:2Δ9,12. The stereochemistry of the 16:2 isomers was assigned based on the known elution order of analogous 18:2 isomers; in both cases, the *trans* isomers elute before the *cis* isomers (Figure 3.6).

Identification of 18:1-9c,12a was achieved by the use of a purified standard during GC-FID and GC-MS analysis. An HPLC-purified 18:1-9c,12a standard was obtained from *Crepis alpina* seed oil which showed the same retention time (~15.5 min) as 18:1-9c,12a produced in yeast expressing either Crep1 or a Crep1 mutant. This purified standard was also analyzed by MS (Figure 3.9A) and shows the same fragmentation pattern as 18:1-9c,12a obtained from yeast cultures expressing Crep1 (Figure 3.9B).

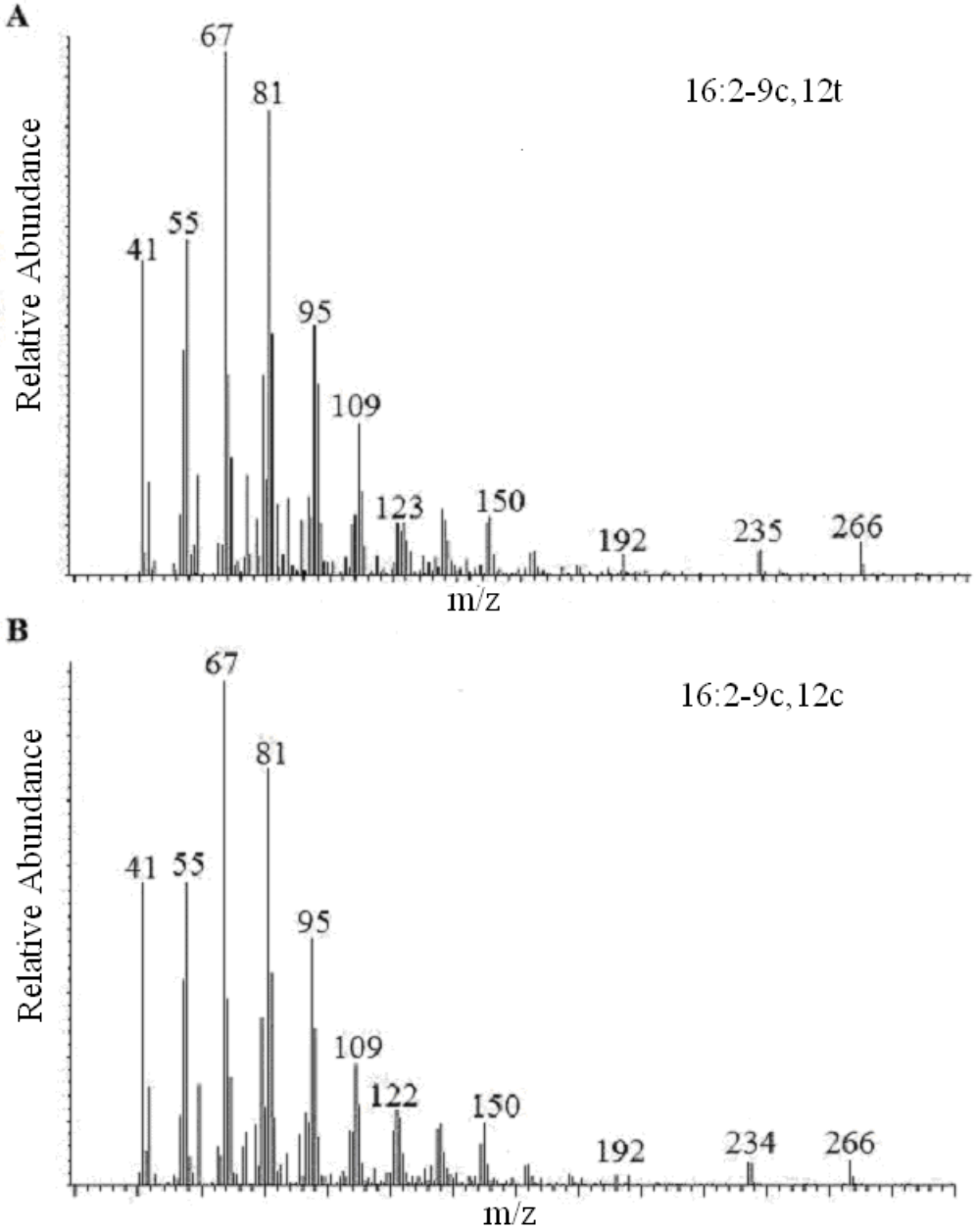

Figure 3.8 MS Spectra for 16:2 Geometric Isomers
MS spectra for the peak labeled 16:2-9c,12c (A) and 16:2-9c,12t (B) seen in Figure 3.6B.

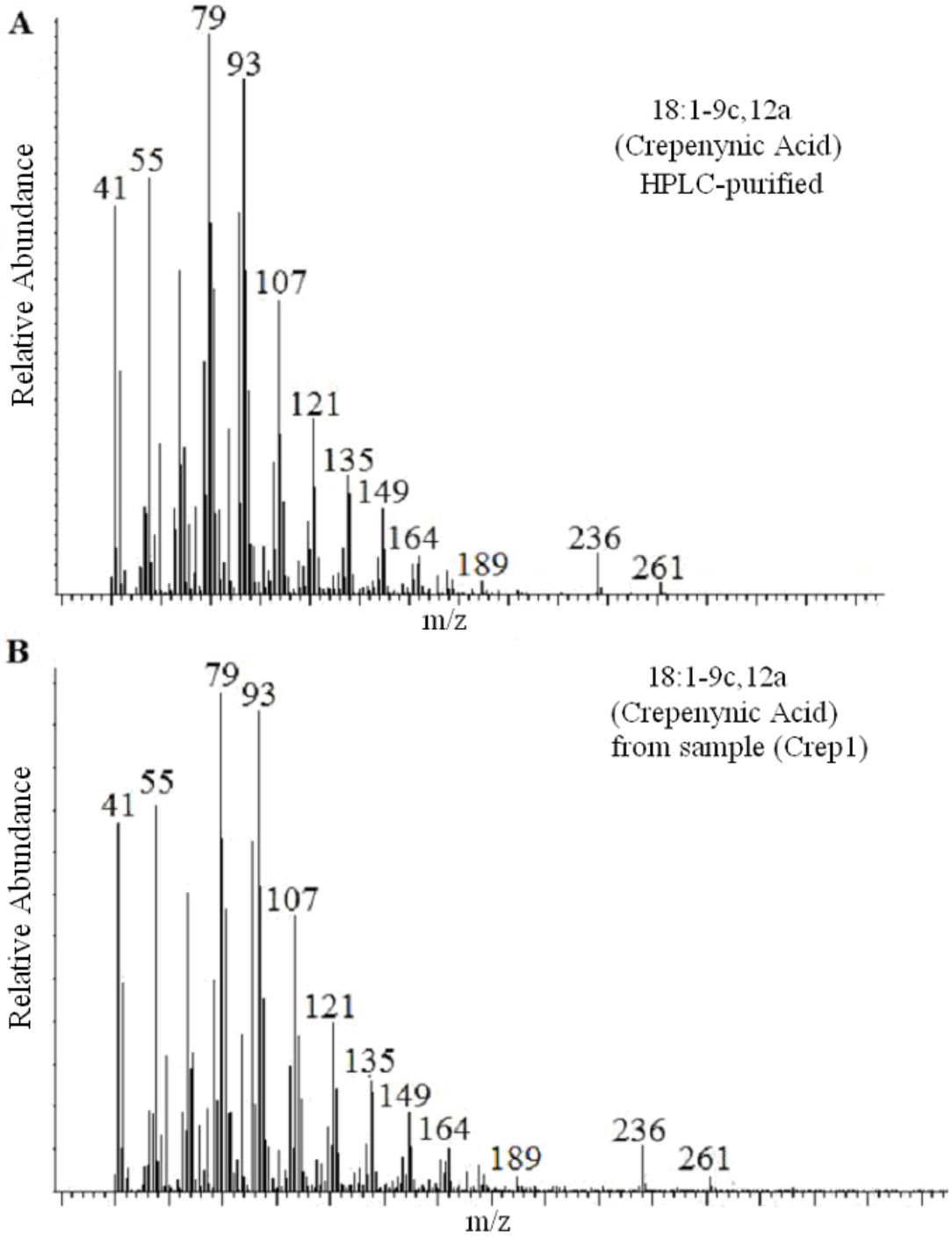

Figure 3.9 MS Identification of 18:1-9c,12a
The MS spectrum for HPLC purified 18:1-9c,12a in (A) compared to the MS spectrum of the peak identified as 18:1-9c,12a in (B) which was obtained from INVSc1 yeast expressing Crep1.

Analysis of 18:1-9c,12a was complicated by the presence of a compound which eluted shortly after 18:1-9c,12a itself (Figure 3.7A). This unknown product was present in all cultures, and was identified by GC-MS as 11-eicosenoic acid (20:1−11c) (Figure 3.10). To validate the presence or absence of 18:1-9c,12a in yeast strains, single ion monitoring was used with m/z = 236, which is found in 18:1-9c,12a-methyl esters (Figure 3.9) but not in 20:1−11c (Figure 3.10).

3.3 Quantitation of Fatty Acid in Yeast Grown in the Presence of 18:2-9c,12c

In this section, the amounts of fatty acids which accumulated in transformed INVSc1 yeast which was grown for 6 days in the presence of 18:2-9c,12c is reported (Table 3.3). These experiments were performed to quantify 18:1-9c,12a and 16:2 geometric isomers in transgenic yeast cultures that expressed Crep1 or Crep1 mutants. 18:2-9c,12c was added to cultures since it acts as a substrate for Crep1 during the generation of 18:1-9c,12a. The addition of 18:2-9c,12c masks the presence of 18:2 isomers that may accumulate in transgenic cultures due to Crep1 activity. Thus 18:2 isomer accumulations is not reported for the experiments covered throughout this section, but will be covered in the next section.

3.3.1 The Accumulation of 18:1-9c,12a in Yeast Cultured in the Presence of 18:2-9c,12c

Yeast strains that had been supplied with 18:2–9c,12c to act as a substrate in the synthesis of 18:1-9c,12a were analyzed by GC-FID. Yeast cultures harboring negative control plasmids (pVT100U) showed no accumulation of 18:1-9c,12a. Yeast cultures that expressed Crep1 accumulated 18:1-9c,12a to levels of 0.199 ± 0.029% TFA (Table 3.3). Relative to the Crep1 strain, yeast strains harboring mutant plasmids showed a drop in the accumulation of 18:1-9c,12a to values between 0.018 - 0.151% TFA (Table 3.3). Yeast strains harboring plasmid pSG004 (Y150F) showed the largest drop in 18:1-9c,12a accumulation to levels of 0.018 ± 0.003% TFA; this represents an 11-fold reduction in 18:1-9c,12a accumulation compared to positive controls. Cultures harboring pSG003 (N146H) accumulated the most 18:1-9c,12a amongst all cultures harboring mutant plasmids; 18:1-9c,12a accumulated to 0.151 ± 0.025% TFA in these cultures. This represents a 24% reduction of 18:1-9c,12a when compared to yeast cultures harboring the unmodified Crep1 (Figure 3.11).

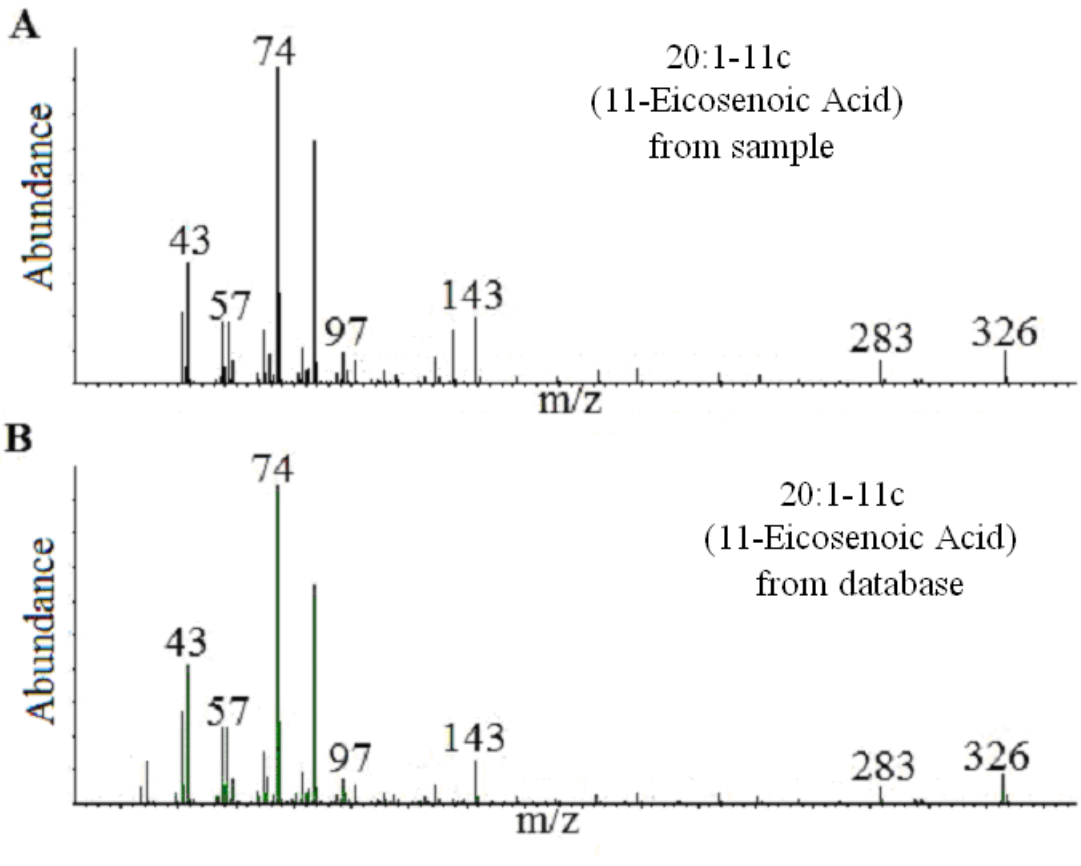

Figure 3.10 MS Identification of 11-Eicosenoic Acid
MS spectra for the product that eluted closely after 18:1-9c,12a (A) shown in Figure 3.7A and of standard 20:1-11c (11-eicosenoic acid) as retrieved from our database (B).

Table 3.3 The Accumulation of Fatty Acids Yeast Cultured in the Presence of 18:2-9c,12c
Fatty acid accumulation observed in INVSC1 harboring respective plasmids. Cultures were grown for 6 days in the presence of 18:2-9c,12c. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate. Results are shown as % TFA.

Plasmid (Mutation)	16:0	16:1-9c	16:2-9c,12t	16:2-9c,12c	18:0	18:1-9c	18:2-9c,12c	18:1-9c12a
pVT100U (- control)	23.98 ±0.18	18.5 ±2.0	0.018 ±.001	ND	7.16 ±1.10	11.1 ±1.5	39.3 ±3.2	ND
pVTCrep1 (unmodified)	24.43 ±0.30	18.0 ±1.9	0.067 ±0.014	ND	7.01 ±0.19	10.5 ±1.4	39.8 ±2.9	0.199 ±0.029
pSG001 (G97A)	24.19 ±0.36	17.9 ±2.1	0.039 ±0.006	ND	7.27 ±0.09	10.9 ±1.6	39.5 ±3.3	0.078 ±0.010
pSG002 (A139S)	24.43 ±0.12	16.6 ±1.1	0.037 ±0.007	ND	7.28 ±0.18	10.0 ±0.8	41.6 ±1.7	0.077 ±0.024
pSG003 (N146H)	24.53 ±0.13	16.5 ±2.0	0.053 ±0.009	ND	7.17 ±0.11	9.9 ±1.5	41.7 ±3.3	0.151 ±0.025
pSG004 (Y150F)	24.16 ±0.18	18.5 ±1.1	0.021 ±0.003	ND	7.18 ±0.05	11.0 ±0.9	39.2 ±1.8	0.018 ±0.003
pSG005 (F183L)	24.13 ±0.26	16.6 ±1.8	0.029 ±0.007	ND	7.08 ±0.08	10.1 ±1.3	42.0 ±2.8	0.042 ±0.016
pSG006 (F183W)	24.04 ±0.37	17.6 ±1.7	0.036 ±0.009	ND	7.06 ±0.13	10.5 ±1.3	40.6 ±2.5	0.073 ±0.016
pSG007 (K194R)	24.21 ±0.38	17.2 ±1.6	0.047 ±0.007	ND	6.92 ±0.31	9.87 ±1.3	41.7 ±2.5	0.080 ±0.014
pSG008 (F259L)	24.17 ±0.33	17.7 ±1.6	0.186 ±0.043	0.034 ±0.008	7.12 ±0.17	10.6 ±1.1	40.0 ±2.3	0.080 ±0.013
pSG009 (H266Q)	24.24 ±0.38	16.9 ±2.0	0.172 ±0.049	ND	7.21 ±0.18	10.2 ±1.5	41.2 ±2.9	0.028 ±0.006
pSG010 (V304I)	24.35 ±0.29	17.5 ±1.6	0.040 ±0.006	0.019 ±0.004	7.08 ±0.10	10.3 ±1.3	40.6 ±2.6	0.101 ±0.015
pSG011 (H306D)	24.29 ±0.25	18.1 ±2.0	0.055 ±0.009	ND	7.16 ±0.19	10.8 ±1.5	39.5 ±3.2	0.059 ±0.007
pSG012 (I317M)	24.27 ±0.14	18.7 ±1.2	0.052 ±0.006	ND	7.20 ±0.12	11.2 ±1.0	38.5 ±2.0	0.132 ±0.017

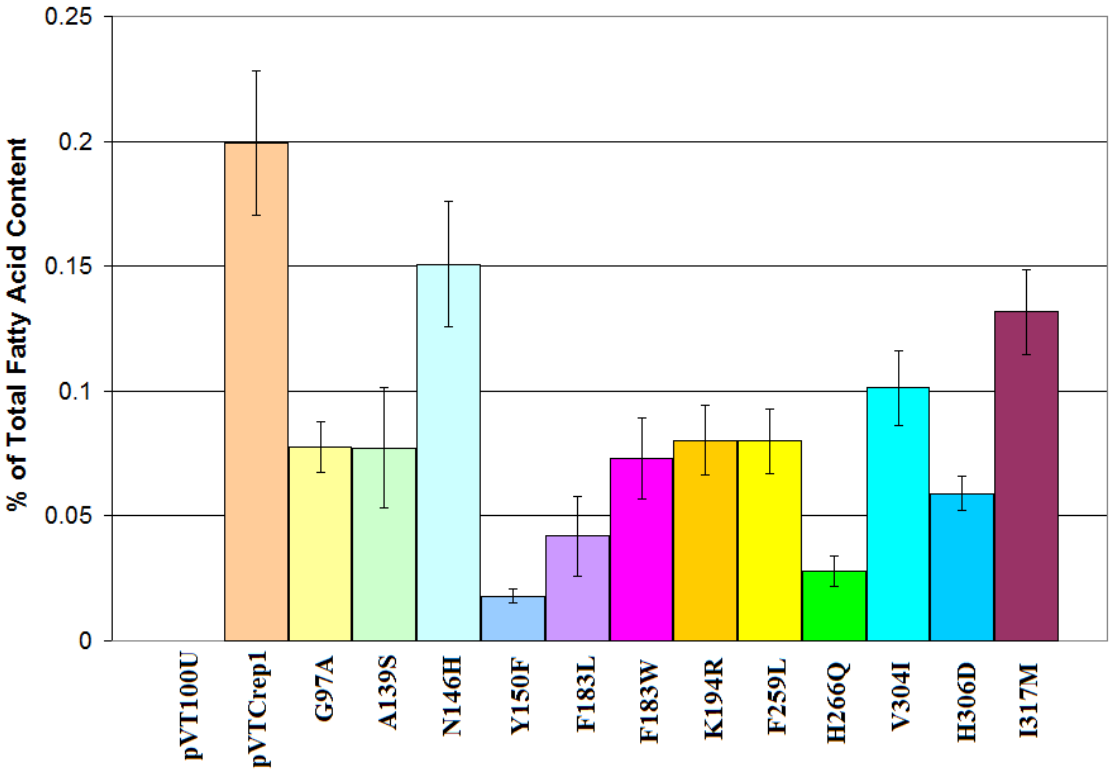

Figure 3.11 Accumulation of 18:1-9c,12a in Yeast Cultured in the Presence of 18:2-9c,12c
A graphical representation indicating the levels of 18:1-9c,12a that accumulated in transformed INVSc1 yeast cultured for 6 days in the presence of 18:2-9c,12c. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate.

3.3.2 The Accumulation of 16:2 Isomers in Yeast Cultured in the Presence of 18:2-9c,12c

16:2 isomers were detected by GC-FID in some transformed INVSc1 strains carrying Crep1 or modified variants when grown in the presence of 18:2-9c,12c, which can be seen in Table 3.3 and Figure 3.7B. INVSc1 strains that were transformed with the empty plasmid pVT100U (negative control) did not accumulate any 16:2-9c,12c isomer and trace amounts of 16:2-9c,12t (Figure 3.7B). Cultures expressing unmodified Crep1 accumulated 16:2-9c,12t to levels of 0.067 ± 0.014% TFA but did not accumulate 16:2-9c,12c isomer in detectable amounts (Figure 3.7B). The largest producers of 16:2-9c,12t were seen in cultures harboring plasmids pSG008 (F259L) and pSG009 (H266Q) where levels of the 16:2 isomer accumulated to 0.186 ± 0.043% TFA and 0.172 ± 0.049% TFA, respectively. This represents an increase of 156-177% of 16:2-9c,12t isomer accumulation when compared to cultures expressing Crep1. Other yeast strains harboring mutant Crep1 constructs produced less 16:2-9c,12t isomer than

yeast cultures expressing Crep1. The smallest amount of 16:2-9c,12t amongst these cultures was detected in yeast strains harboring pSG004 where levels accumulated to 0.021 ± 0.003% TFA. This represents a 67% decrease of 16:2-9c,12t isomer accumulation when compared to cultures expressing Crep1 (Figure 3.12).

Of all cultures analyzed, only 2 transformed strains accumulated 16:2-9c,12c to detectable amounts. Strains harboring pSG008 (F259L) showed 0.034 ± 0.008% TFA of 16:2-9c,12c (Figure 3.6), where strains harboring pSG010 (V304I) accumulated lesser amounts of 0.019 ± 0.004% TFA (Table 3.3).

3.4 Quantitation of Fatty Acid in Yeast Grown in the Absence of 18:2-9c,12c

This section deals with the amounts of fatty acids that accumulated in transformed INVSc1 yeast cultured for 6 days in the absence of 18:2-9c,12c. These experiments were performed to quantify 18:2Δ9,12 and 16:2Δ9,12 geometric isomers in transgenic yeast cultures that expressed Crep1 or Crep1 mutants. Since 18:2-9c,12c was not added to these cultures, there was not enough substrate available for Crep1 (and mutants) to produce 18:1-9c,12a. No 18:1-9c,12a was detected by GC-MS in any yeast culture that was not provided an external source of 18:2-9c,12c. The lack of 18:2-9c,12c allows for the detection of 18:2Δ9,12 as well as 16:2Δ9,12 isomers that accumulated in transgenic cultures due to Crep1 desaturase activity (Table 3.4).

3.4.1 The Accumulation of 18:2-9c,12c in Yeast Cultures in the Absence of 18:2-9c,12c

18:2-9c,12c was detected by GC-FID in transformed INVSc1 strains that were grown for 6 days in the absence of 18:2-9c,12c (Figure 3.13). INVSc1 strains that were transformed with the empty plasmid pVT100U (negative control) accumulated trace amounts of the 18:2 isomer to levels of 0.010 ± 0.001% TFA. Cultures harboring pVTCrep1 accumulated levels of 0.039 ± 0.001% TFA. In regards to cultures expressing Crep1 mutants, cultures harboring pSG005 (F183L) contained the least amount of 18:2-9c,12c with levels of 0.013 ± 0% TFA, which is similar to negative controls. Cultures harboring pSG008 (F259L) produced the most 18:2-9c,12c with levels accumulating to 0.239 ± 0.017% TFA, which is an ~5-fold increase compared to cultures expressing Crep1.

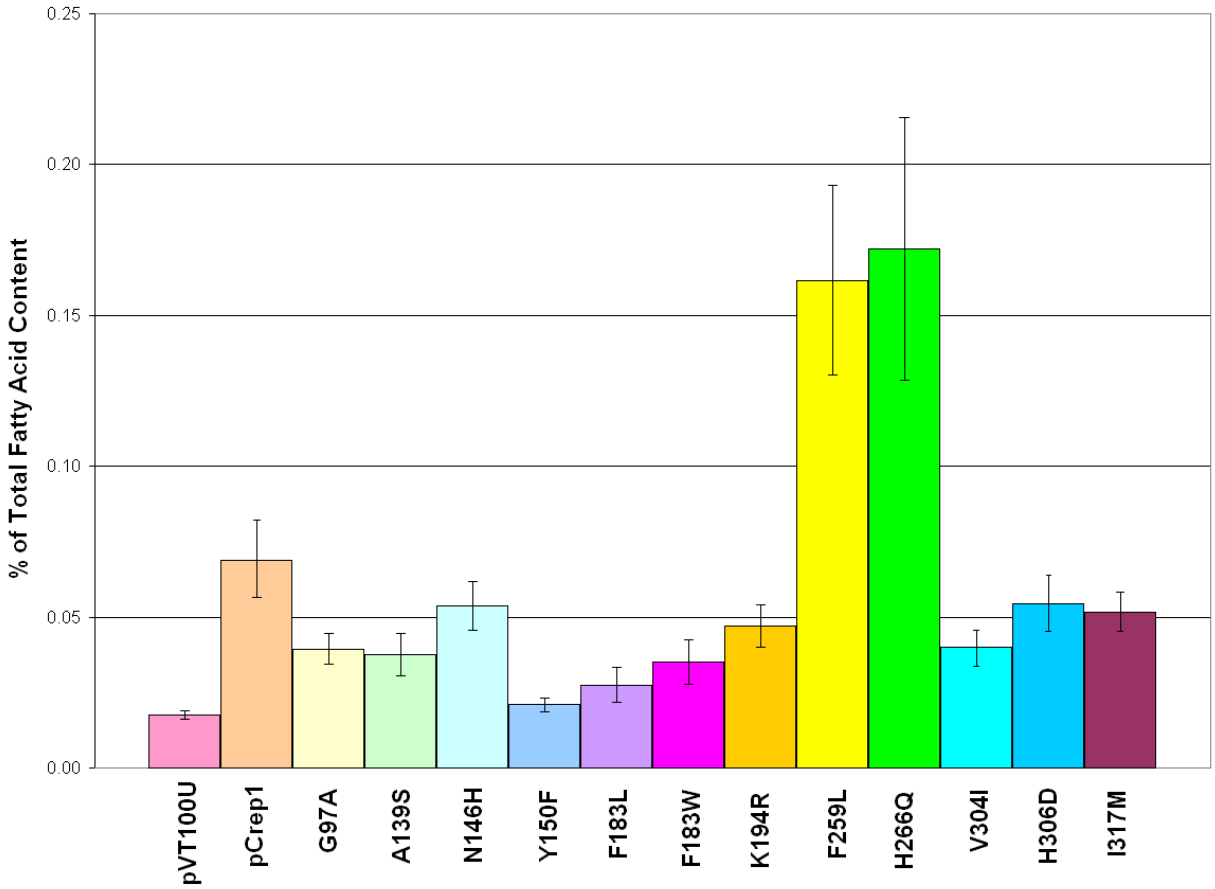

Figure 3.12 Accumulation of 16:2-9c,12t in Yeast Cultured in the Presence of 18:2-9c,12c
A histogram indicating the levels of 16:2-9c,12t that accumulated in transformed INVSc1 yeast cultures that were grown for 6 days in the presence of 18:2-9c,12c. Results represent the means +/- standard error where n=3 independent experiments conducted in triplicate.

Table 3.4 The Accumulation of Fatty Acids in Yeast Cultured in Absence of 18:2-9c,12c
Fatty acid accumulation observed in INVSc1 harboring respective plasmids. Cultures were grown for 6 days in the absence of 18:2-9c,12c. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate. Values represent the % TFA.

Plasmid (Mutation)	16:0	16:1-9c	16:2-9c,12t	16:2-9c,12c	18:0	18:1-9c	18:2-9c,12t	18:2-9c,12c
pVT100U (- control)	18.92 ±0.18	45.74 ±0.22	ND	ND	6.48 ±.09	28.85 ±0.06	ND	0.010 ±0.001
pVTCrep1 (unmodified)	19.54 ±0.22	45.34 ±0.08	0.107 ±0.002	ND	6.73 ±0.11	28.18 ±0.16	0.056 ±0.002	0.039 ±0.001
SG001 (G97A)	19.37 ±0.26	45.23 ±0.23	0.072 ±0.009	ND	6.73 ±0.15	28.52 ±0.13	0.043 ±0.005	0.036 ±0.004
SG002 (A139S)	19.26 ±0.27	45.34 ±0.12	0.042 ±0.003	ND	6.56 ±0.044	28.75 ±0.19	0.025 ±0.001	0.022 ±0.002
SG003 (N146H)	19.29 ±0.20	45.23 ±0.05	0.094 ±0.012	ND	6.71 ±0.07	28.59 ±0.18	0.053 ±0.007	0.037 ±0.004
SG004 (Y150F)	19.11 ±0.33	45.48 ±0.014	0.015 ±0	ND	6.62 ±0.09	28.69 ±0.29	0.052 ±0.002	0.037 ±0.001
SG005 (F183L)	19.58 ±0.43	45.19 ±0.06	0.021 ±0.002	ND	6.69 ±0.10	28.50 ±0.29	0.009 ±0.001	0.013 ±0
SG006 (F183W)	19.38 ±0.31	45.48 ±0.37	0.036 ±0.002	ND	6.59 ±0.13	28.42 ±0.09	0.057 ±0.003	0.037 ±0.001
SG007 (K194R)	19.26 ±0.21	45.14 ±0.24	0.064 ±0.002	ND	6.60 ±0.03	28.89 ±0.20	0.033 ±0.002	0.027 ±0.001
SG008 (F259L)	19.38 ±0.16	45.95 ±1.04	0.301 ±0.027	0.062 ±0.005	6.27 ±0.47	27.70 ±0.77	0.111 ±0.008	0.239 ±0.017
SG009 (H266Q)	19.46 ±0.42	44.97 ±0.09	0.280 ±0.028	ND	6.65 ±0.11	28.44 ±0.25	0.156 ±0.015	0.035 ±0.003
SG010 (V304I)	19.73 ±0.63	45.07 ±0.15	0.030 ±0.003	0.021 ±0.002	6.66 ±0.19	28.43 ±0.51	0.009 ±0.002	0.047 ±0.004
SG011 (H306D)	19.54 ±0.34	45.24 ±0.18	0.041 ±0.001	ND	6.56 ±0.09	28.60 ±0.18	0.010 ±0.002	0.021 ±0
SG012 (I317M)	19.28 ±0.37	45.34 ±0.06	0.066 ±0.001	ND	6.48 ±0.13	28.78 ±0.30	0.037 ±0.001	0.029 ±0

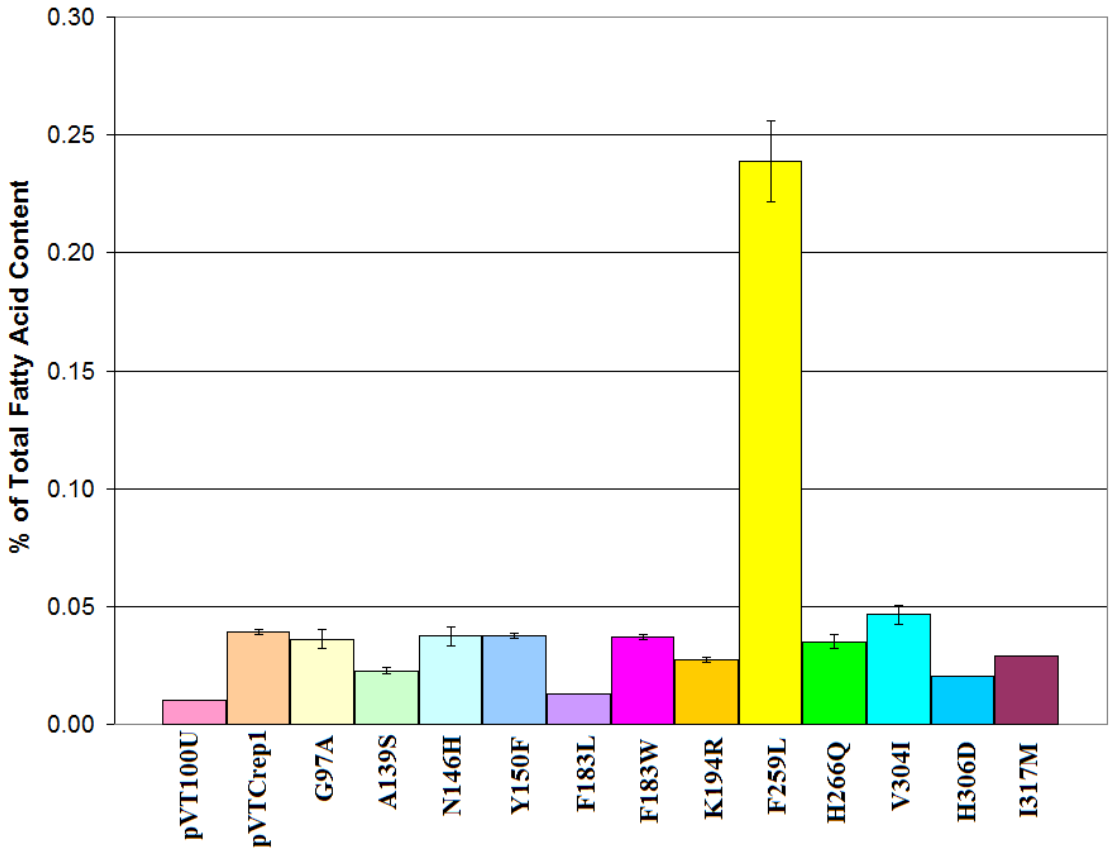

Figure 3.13 Accumulation of 18:2-9c,12c in Yeast Cultured in the Absence of 18:2-9c,12c
A histogram indicating the levels of 18:2-9c,12c that accumulated in transformed INVSc1 yeast cultured for 6 days in the absence of 18:2-9c,12c. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate.

3.4.2 The Accumulation of 18:2-9c,12t in Yeast Cultures in the Absence of 18:2-9c,12c

The percentage of 18:2-9c,12t was determined by GC-FID in transformed INVSc1 strains that were grown for 6 days in the absence of 18:2-9c,12c (Figure 3.14). INVSc1 strains that were transformed with the empty plasmid pVT100U (negative control) did not accumulate any levels of 0.056 ± 0.002% TFA. The least amount of 18:2-c9,12t detected (other than in negative 0.009 ± 0.001% TFA. Larger quantities of 18:2-9c,12t were detected in yeast harboring pSG008 (F259L) with levels of 0.111 ± 0.008% TFA, an increase of 98% compared to Crep1 cultures. The greatest amounts of 18:2-9c,12t was seen in cultures harboring pSG009 (H266Q) with levels of 0.156 ± 0.015% TFA, an increase of 179% when compared to cultures expressing Crep1.

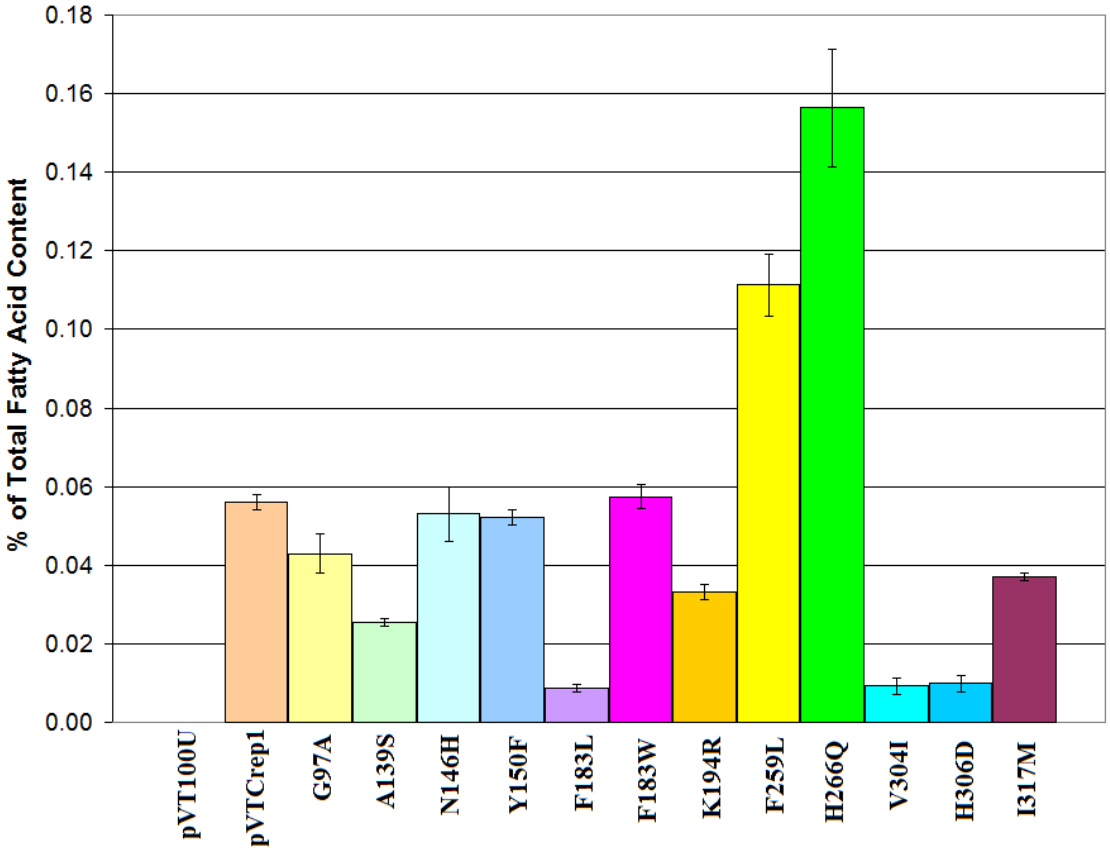

Figure 3.14 Accumulation of 18:2-9c,12t in Yeast Cultured in the Absence of 18:2-9c,12c
A histogram indicating the levels of 18:2-9c,12t that accumulated in transformed INVSc1 yeast cultured for 6 days. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate.

3.4.3 The Accumulation of 16:2–9c,12c and 16:2-9c,12t in Yeast Cultured in the Absence of 18:2-9c,12c

16:2 isomers were detected by GC-FID in transformed INVSc1 strains when grown in the absence of 18:2-9c,12c (Figure 3.15). Although greater amounts of 16:2 isomers were detected in cultures not provided with 18:2-9c,12c when compared to cultures that had been grown in the presence of 18:2-9c,12c, their accumulation patterns were remarkably similar. Neither 16:2-9c,12t nor 16:2-9c,12c isomers were detected in INVSc1 strains that were transformed with the empty plasmid pVT100U (negative control) when cultured in the absence of 18:2-9c,12c. Cultures expressing unmodified Crep1 accumulated 16:2-9c,12t to levels of 0.107 ± 0.002% TFA but did not accumulate any 16:2-9c,12c. The largest producers of 16:2-9c,12t were seen

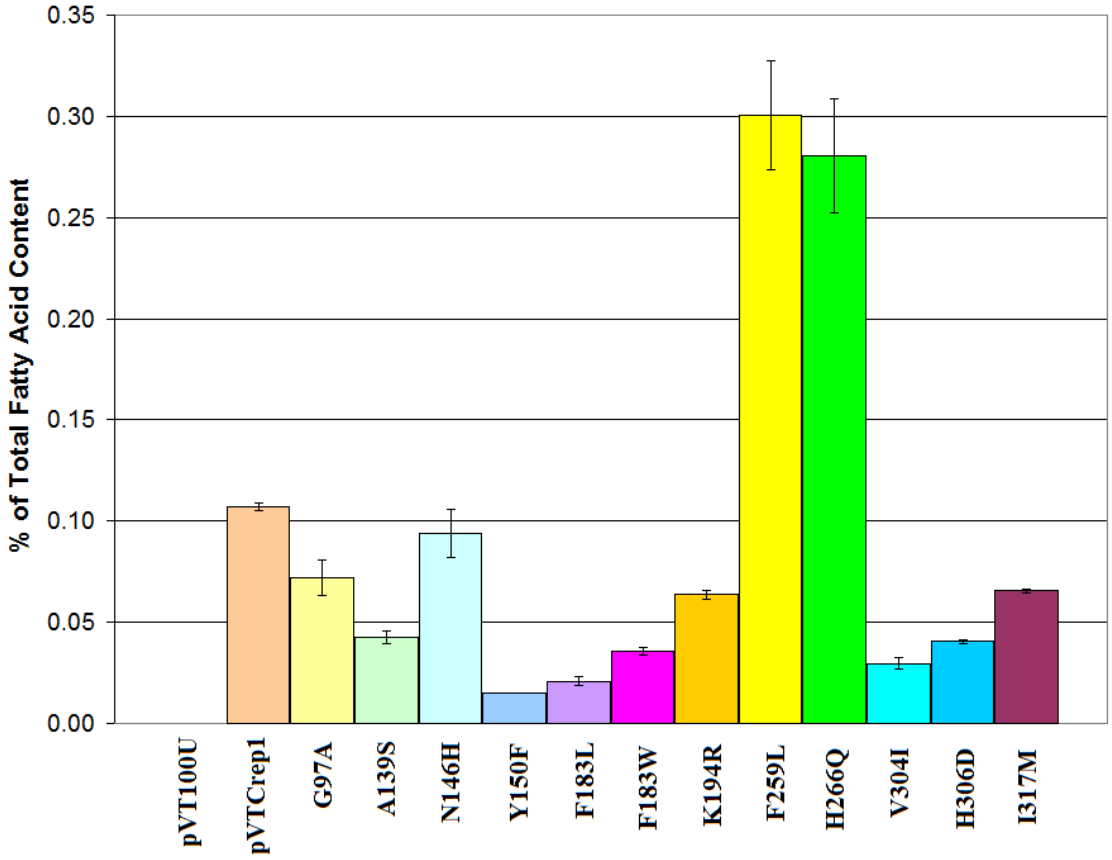

Figure 3.15 Accumulation of 16:2-9c,12t in Yeast Cultured in the Absence of 18:2-9c,12c
A histogram indicating the levels of 16:2-9c,12t that accumulated in transformed INVSc1 yeast cultured for 6 days in the absence of 18:2-9c,12c. Results represent the means ± standard errors (n=3) from 3 independent experiments performed in triplicate.

in cultures harboring plasmids pSG008 (F259L) and pSG009 (H266Q) where levels of the 16:2 isomer accumulated to 0.301 ± 0.027% TFA and 0.280 ± 0.028% TFA, respectively. This represents an increase of 162-181% of 16:2-9c,12t isomer accumulation when compared to cultures expressing Crep1. In congruence with earlier experiments, the smallest amount of 16:2-9c,12t was detected in yeast strains harboring pSG004 (Y150F) where levels accumulated to 0.015% TFA. This represents a 86% decrease of 16:2-9c,12t accumulation when compared to cultures expressing Crep1.

The 16:2-9c,12c isomer was only detected in two transformed yeast strains. Strains harboring pSG008 (F259L) showed 0.062 ± 0.005% TFA of 16:2-9c,12c, whereas strains harboring pSG010 (V304I) accumulated amounts of 0.021 ± 0.002% TFA (Table 3.4).

3.5 Analysis of Ratios Used to Determine the Effects of Mutations in Crep1

While comparing the data from relative amounts of fatty acids indicates potential key target residues within Crep1, some product ratios are instrumental in determining how specific amino acid residues affect activity. Product ratios are used to determine mutational effects on: 1) Crep1 acetylenase activity to desaturase activity; 2) *cis/trans* ratios for desaturase activity (*i.e.* if there is a preference for the production of cis or trans isomers in mutant constructs); and 3) Crep1 substrate selectivity (*i.e.* if there is a preference for 16:1-9c or 18:1-9c during desaturation).

3.5.1 Ratios Used to Determine Desaturase/Acetylenase Activity

To determine whether mutant constructs had increased desaturase activity relative to acetylenase activity, product ratios considering the accumulation of 18:2-9c,12c and 18:1-9c,12a in transgenic yeast cultures were calculated (Table 3.5). Average amounts of 18:2-9c,12c that accumulated in each strain grown in the absence of 18:2-9c,12c were compared to the average amounts of 18:1-9c,12a that accumulated in the same strain when grown in the presence of 18:2-9c,12c. These product ratios allow for the determination of how a mutation within Crep1 affects relative desaturase and acetylenase activities. Product ratios were also compared to the desaturation/acetylenation ratio in yeast strains expressing unmodified Crep1 and are reported as a fold increase.

Three Crep1 mutant constructs show an increase in desaturation when compared to unmodified Crep1: pSG004 (Y150F) shows a 10.5 fold increase in desaturase activity relative to acetylenase activity; pSG008 (F259L) shows a 15 fold increase in desaturase activity; pSG009 (H266Q) showed a 6.4 fold increase in desaturase vs. acetylenase activity when compared to the positive control strains expressing Crep1. Product ratios were compiled from the means and standard error (n=3) from three independent experiments conducted in triplicate.

3.5.2 Ratios Used to Determine *cis/trans* Desaturase Activity

To determine if point mutations affect Crep1 desaturase activity so that there is a preference for *cis* or *trans* isomer formation, the total *cis* isomer accumulation was compared to the total *trans* isomer formation in transgenic yeast cultures. Average amount of 16:2-9c,12c and 18:2-9c,12c which accumulated were summed and compared to total amounts of (summed) 16:2-9c,12t

Table 3.5 Product Ratios Used to Determine the Effects of Mutations on Crep1

Three different ratios were used to determine the effect of point mutations on Crep1 activity. Product ratios used to define the relationship between desaturation and acetylenation were based on the average amounts of 18:2-9c,12c compared to 18:1-9c,12a which accumulated in Crep1 and Crep1 mutant cultures. These ratios are listed below the 'desaturation/acetylenation' heading. The preference for *cis* isomer formation compared to *trans* isomer production is calculated by considering the total amount of 16:2-9c,12c and 18:2-9c,12c compared to total amounts of 16:2-9c,12t and 18:2-9c,12t and is found under the 'total *cis*/total *trans*' column. Substrate selectivity was determined by considering the total amounts of 16:2Δ9,12 isomers produced compared to 18:2Δ9,12 isomers produced and is found under the total '16:2/total 18:2' column. All ratios are compared to ratios obtained from yeast expressing unmodified Crep1 and are listed in the 'fold increase' column following respective ratios.

Plasmid (Mutation)	Desaturation / Acetylenation	Fold Difference	Total *cis* / Total *trans*	Fold Difference	Total 16:2 / Total 18:2	Fold Difference
pVTCrep1 (unmodified)	0.20 ± 0.03	1	0.239 ± 0.008	1	1.13 ± 0.04	1
pSG001 (G97A)	0.46 ± 0.08	2.4	0.313 ± 0.052	1.3	0.91 ± 0.15	0.8
pSG002 (A139S)	0.29 ± 0.09	1.5	0.328 ± 0.036	1.4	0.89 ± 0.09	0.8
pSG003 (N146H)	0.25 ± 0.05	1.3	0.252 ± 0.042	1.1	1.04 ± 0.18	0.9
pSG004 (Y150F)	2.06 ± 0.35	10.5	0.552 ± 0.022	2.3	0.17 ± 0.01	0.1
pSG005 (F183L)	0.31 ± 0.12	1.6	0.433 ± 0.043	1.8	0.95 ± 0.10	0.8
pSG006 (F183W)	0.51 ± 0.11	2.6	0.398 ± 0.024	1.7	0.38 ± 0.03	0.3
pSG007 (K194R)	0.34 ± 0.06	1.7	0.278 ± 0.015	1.2	1.07 ± 0.06	0.9
pSG008 (F259L)	2.99 ± 0.53	15.2	0.731 ± 0.082	3.1	1.04 ± 0.12	0.9
pSG009 (H266Q)	1.25 ± 0.29	6.4	0.08 ± 0.01	0.3	1.5 ± 0.2	1.3
pSG010 (V304I)	0.47 ± 0.08	2.4	1.74 ± 0.27	7.3	0.91 ± 0.13	0.8
pSG011 (H306D)	0.36 ± 0.04	1.8	0.412 ± 0.024	1.7	1.32 ± 0.09	1.2
pSG012 (I317M)	0.22 ± 0.03	1.1	0.282 ± 0.005	1.2	1.00 ± 0.02	0.9

and 18:2-9c,12t which accumulated in each yeast strain. Product ratios were also compared to *cis/trans* product ratio determined in yeast expressing unmodified Crep1 and are reported as a fold increase (Table 3.5).

With regard to the total *cis* to total *trans* product ratios, 3 mutant constructs increased production of *cis* isomers when compared to Crep1. These constructs are pSG004 (Y150F) which shows a 2.3 fold increase, pSG008 (F259L) which indicates a 3.1 fold increase, and pSG010 (V304I) which had a 6.4 fold increase. These constructs produced more *cis* isomers than *trans* isomers when compared to Crep1 controls.

A decreased *cis* to *trans* product ratio was seen in only one mutant construct, pSG009 (H266Q). Yeast strains harboring this plasmid showed a 70% decrease in *cis/trans* product accumulation when compared to Crep1 controls which indicates a preference for the *trans* isomer to be formed during desaturation.

3.5.3 Ratios Used to Determine Substrate Selectivity

To determine if Crep1 mutants preferred 16:1–9c or 18:1–9c as a substrate during desaturation, the 16:2/18:2 product ratios were calculated and compared (Table 3.5). The summed amounts of 16:2 isomers were compared to summed 18:2 isomers for each yeast strain grown in the absence of 18:2-9c,12c. 16:2/18:2 product ratios were also compared to controls expressing unmodified Crep1 and are reported as a fold increase.

Table 3.5 shows that two mutant constructs appear to prefer 18:1–9c as a substrate: pSG004 (Y150F) showed a 90% decrease of 16:2/18:2 product accumulation when compared to Crep1; pSG006 (F183W) showed a 20% decrease in 16:2/18:2 product accumulation when compared to Crep1 controls. Other mutant constructs did not appear to substantially affect substrate selectivity.

4. DISCUSSION

In this work the structure/function relationship between FAD2s and acetylenases was considered by examining the conserved amino acid residues which occur uniquely in either class of enzymes. Such amino acid residues were targeted for site-directed mutagenesis within the acetylenase Crep1, exchanging native amino acids in Crep1 with residues normally found in FAD2s. The effects that these mutations had on the relative activity of Crep1 were observed after expressing the mutated enzymes in INVSc1 and analyzing the product ratios of the transformed cultures by GC-FID and GC-MS. By assessing different product ratios, the effect mutations had on relative Crep1 activity could be divided into three different categories: relative acetylenase to desaturase activity, stereoselectivity, and substrate selectivity.

4.1 Sequence Similarity and Choice of Mutants

Conservation of amino acids in the selected FAD2 and acetylenase sequences were compared by using CPDL software. CPDL compares the conservation of amino acids between two subsets of protein sequences and reports the differences between these two subsets. CPDL also compares amino acid properties, such as size and hydrophobicity, which are conserved in either group of enzymes. Only the CPDL results that indicated conserved amino acids and not conserved amino acid properties for site-directed mutagenesis were regarded. A rational approach in determining which amino acids most likely differentiate FAD2 and acetylenase activity was achieved by using CPDL. This approach is based on the neutral theory of molecular evolution (NTOME) which holds that molecular evolution is dominated by genetic drift and not solely by selective adaptation (Kimura, 1983). The rate of molecular evolution is constant, an effect not explained by adaptation, and is proportional to the rate of mutation. The NTOME considers that most fixed mutations that occur at the molecular level are neutral in nature, and do not affect the active site and/or other critical sites within an enzyme. Therefore a mutation that causes the substitution/deletion of an amino acid residue within a protein that is not involved in the protein's direct function should not affect or influence the overall fitness of the allele, and is therefore inconsequential and not selected for. In contrast, an amino acid residue which is involved in the functionality of a protein is required for the allele's overall fitness and may be selected for at the genetic level. The NTOME is the basis for modern protein engineering which has been recently discussed by Shanklin, 2008.

It was determined that strict amino acid conservation differed at 11 key positions when considering FAD2s and acetylenases. CPDL results also indicated other sites between the two groups of enzymes which had differences in properties, but no strict conservation of amino acids occurred. Although these other sites may affect the structure and function of FAD2 and acetylenase enzymes, they were considered to be secondary to the identified conserved amino acid differences and were not regarded furthermore in this study. It was decided that the 11 amino acid locations were of primary importance and that these targets were the priority for further experimentation.

4.2 Expression of pVTCrep1 and pVT100 in Yeast

The production of 18:1-9c,12a, as well as 16:2 and 18:2 *cis/trans* isomers in transgenic yeast was successfully accomplished in these studies. Cultures expressing pVTCrep1 were used to represent the Crep1 positive control throughout this work, and cultures expressing pVT100U (empty plasmid) were used as the negative control. 18:1-9c,12a was detected in yeast cultures harboring pVTCrep1, which accumulated to 0.199% TFA when grown in the presence of the substrate, 18:2-9c,12c. Initial attempts from earlier research to produce 18:1-9c,12a in transgenic yeast successfully generated up to 0.3% of total peak area using GC-FID (Lee *et al.*, 1998). Later studies which also expressed pVTCrep1 in yeast were more successful. Carlsson *et al.*, 2004, reported 18:1-9c,12a accumulation to 0.53% TFA when transgenic yeast was grown in the presence of 18:2-9c,12c. Differences between our values and those reported by Lee *et al.,* 1998, and Carlsson *et al.,* 2004, may be attributed to the different yeast strain used to express pVTCrep1, the method by which fatty acids were prepared for FID-GC analysis, and by differences in the fatty acid profiles used by each laboratory. 18:1-9c,12a was not detected in negative controls.

In addition to 18:1-9c,12a, the desaturation products that accumulated in transgenic cultures when grown in the presence of 18:2-9c,12c were also considered. When cultures were fed 18:2-9c,12c, no 18:2 fatty acid could be considered as a direct product of the transgenic yeast since amounts were masked by the addition of the substrate. Although 18:2 products could not be considered in these cultures, 16:2-9c,12t product was detected in cultures expressing Crep1. 16:2-9c,12c was not detected in cultures harboring pVTCrep1, but 16:2-

9c,12t did accumulate to 0.067% TFA. There exists no previous report of 16:2 accumulations with regard to Crep1.

When cultures were grown in the absence of substrate (18:2-9c,12c), 18:2 and 16:2 geometric isomers were successfully detected. Cultures harboring pVTCrep1 accumulated 18:2-9c,12c to levels of 0.039% TFA. This amount represents the product of a FAD2-like desaturation of endogenous 18:1-9c. The amount of 18:2-9c,12c detected is less than those reported by previous investigators (Carlsson *et al.,* 2004) who report an accumulation of 18:2-9c,12c to 0.18% TFA. Again, these differences may be due to the different yeast strain used by both laboratories, the differences in culturing, sample preparation, and fatty acid profiles used. Slight amounts of 18:2-9c,12c to levels of 0.010% TFA were also detected in negative controls. These slight amounts of 18:2-9c,12c, which were very close to the detection limits of our instruments (~0.005%), should not have appeared and are possibly due to impurities found in the media itself. Carlsson *et al.*, 2004, did not detect any such product in their negative controls. The amounts of 18:2-9c,12c observed in transgenic cultures were constant throughout the experiments, and the increased production of 18:2-9c,12c in positive controls does indicate that Crep1 possesses a FAD2-like desaturation function when compared to our negative controls.

18:2-9c,12t was also detected in yeast expressing pVTCrep1, but was not detected in negative controls. The 18:2-9c,12t is not a typical FAD2 desaturation product and instead represents a diverged FAD2-like reaction. The amount of 18:2-9c,12t detected in this work accumulated to 0.056% TFA. This amount is smaller than that reported by Carlsson *et al.*, 2004, which reported an accumulation to 0.47% TFA.

The amounts of 16:2 isomers have not previously been reported, but prove valuable for comparison of cultures which have and have not been grown in the presence of 18:2-9c,12c. 16:2-9c,12c was not detected in positive controls when grown in the absence or presence of 18:2-9c,12c. The 16:2-9c,12t isomer accumulated to 0.107% TFA, a greater amount than that seen in cultures grown in the presence of 18:2-9c,12c. This increase in 16:2-9c,12t is probably due to a proportional effect seen in the TFA profile, since these cultures lacked the added 18:2-9c,12c which offset the proportional levels of all other fatty acids detected in cultures. The production of 16:2-9c,12t in Crep1 expressing cultures, whether they were grown in the

presence or absence of 18:2-9c,12c confirms the *trans* desaturation activity of the Crep1 enzyme. No 16:2 isomers were detected in negative controls.

4.3 Expression of Crep1 Mutants in Yeast

The activity of Crep1 and mutants derived from Crep1 cannot be measured directly *in vitro* since these proteins are incalcitrant to purification methods due to their membrane-association. Furthermore, the protein expression levels of Crep1 were not measured and therefore the relative amounts of fatty acids that accumulated may be deceiving, although at times are instrumental as complementary data. Thus, the effect that point mutations had on the relative activity of Crep1 was considered by comparing product ratios for specific fatty acids in positive controls with that found in Crep1 mutant expressing cultures. The use of product ratios has been well established in previous studies which consider the functionality of desaturases (Broun *et al.,* 1998a; Broadwater *et al.,* 2002). As well it is important to consider how point mutations affect the relative activity of Crep1 and to designate the differences with respect to chemoselectivity, substrate selectivity, and stereoselectivity. These different activities of Crep1 are individually discussed in the following subsections.

4.3.1 Chemoselectivity

The preference for Crep1 mutants to insert either a double bond or a triple bond at the Δ12 position is discussed in this section. The relative desaturase and acetylenase activities were considered by comparing the product ratios of total 18:2-9c,12c to total 18:1-9c,12a accumulation in transgenic cultures. When compared to positive controls, the desaturation/acetylenation ratio of F259L mutants showed the greatest change with a ~15 fold increase. Other mutations also showed a large increase in this respect, including Y150F and H266Q which displayed 10.5 and 6.4 fold increase respectively (Table 4.1).

Although there were other fluctuations seen in product ratios throughout the array of mutant constructs, no other mutation increased Δ12 desaturation as strongly as F259L. Based on the desaturase/acetylenase product ratio, position F259L appears to be the most important determinant tested regarding the chemoselectivity of Crep1. The F259L mutation had the effect of partially converting the Crep1 acetylenase to a FAD2-like desaturase.

The Y150F point mutation also caused a large change in acetylenation to desaturation suggesting that tyrosine at this position plays a key role in the acetylenase functionality of Crep1. Y150F also appears to be involved in substrate recognition (discussed below under substrate selectivity) capable of desaturation of the 18:1-9c substrate, but not as effectively in 16:1-9c desaturation. This strongly suggests that Y150 is involved in the active site geometry and/or substrate binding cavity.

Relative activity ratios obtained from yeast harboring pSG009 (H266Q) also showed an increase of desaturation over acetylenation when compared to positive controls. This increase in relative desaturase activity over acetylenase activity is coupled to a decrease in 18:1-9c,12a accumulation, whereas 18:2-9c,12c accumulation remains relatively the same as that seen in positive controls. This suggests that although H266Q is a strong determinant of relative acetylenase activity, it does not attenuate relative *cis* desaturase activity.

4.3.2 Stereoselectivity

Amino acid changes that affect the stereoselectivity of Crep1 are examined by considering the product ratios of total 18:2 and 16:2 12-*cis* isomers to the total 18:2 and 16:2 12-*trans* isomers. Total *cis* to *trans* product ratios indicate that either point mutations at V304I or F259L result in an increased stereoselective preference for the 12-*cis* isomer to be formed when compared to controls, whereas a point mutation at H266Q increases preference for 12-*trans* isomer production (Table 4.1).

These changes indicate that the location of these mutations play an important role in substrate binding and active site geometry. The determinant of whether a *cis* or *trans* isomer is produced is believed to be based on the conformation that the substrate molecule possesses (*cisoid* vs *transoid*) when introduced to the active site (Carlsson *et al.,* 2004). This premise is based on observations that hydrogen abstraction occurs in a step-wise manner initially at the C12 position (slow) followed by another hydrogen abstraction at C13 (fast) (Reed *et al.,* 2003). The intermediate in this 2-step reaction has never been isolated, leading Carlsson *et al.*, 2004 to suggest that a change in conformation during the reaction is unlikely, and that the substrate must already possess a specific conformation which will determine the reaction outcome. In the

Table 4.1 Crep1 Targeted Amino Acids Showing the Greatest Change in Product Ratio when Compared to Positive Controls

This table highlights the 4 point mutations which affected Crep1 relative activity the greatest. Values represent the fold increase or decrease in product ratio compared to the positive control (where the ratio in the positive control is always = 1).

Crep1 Mutation	RATIO *cis* Desaturation Acetylenation	RATIO Total *cis* Total *trans*	RATIO Total 16:2 Total 18:2
Y150F	10.5	2.3	0.1
F259L	15.2	3.1	0.9
H266Q	6.4	0.3	1.3
V304I	2.4	7.3	0.8

case of the *cis* isomer, an abstraction of the pro-R hydrogen at both carbons (first at C12 then quickly afterwards at C13) is required, whereas during the *trans* isomer formation, a pro-R hydrogen abstraction at C12 is followed by a pro-S hydrogen abstraction at C13. The production of either a *cis* or *trans* isomer requires that respectively, the pro-R or pro-S hydrogen of C13 is in close proximity to the active site (Figure 4.1). Thus, the amino acid changes which affect the stereoselectivity of Crep1 should also affect the structure of the substrate-binding cavity or possibly the active site geometry itself.

The accumulation patterns of *cis* and *trans* isomers show that although *cis* isomer formation is preferred with the F259L mutation, both *cis* and *trans* isomer production is increased when compared to controls. The case for substrate conformation does not seem to apply for this mutation, which affects more the desaturase activity than solely the stereoselectivity of Crep1. Conversely, the affect that V304I has on stereoselectivity is coupled with a diminished ability to produce both 16:2-9c,12t and 18:2-9c,12t and a slight increase in the amount of 16:2-9c,12c and 18:2-9c,12c when compared to controls, which suggests that a substrate binds preferably in a *cisoid* conformation, or at least, that a pro-R hydrogen abstraction at C13 predominates.

The H266Q mutation also strongly affects Crep1 stereoselectivity, although in this case, the production of the *trans* isomer is strongly preferred over the *cis* isomer. The stereoselective preference caused by the H266Q mutation, coupled to the increased desaturation/acetylenation

ratio discussed earlier (see section 4.3.1), indicate that substrate binding cannot appropriately accommodate a *cis* isomer, whether it is a product, or in the case of 18:1-9c,12a production, a substrate. According to these results, a mutation at H266Q in Crep1 generates a serendipitously engineered Δ12 *trans* desaturase.

4.3.3 Substrate selectivity

Some point mutations in Crep1 led to changes in the total 16:2 isomers to total 18:2 isomers ratio. The differences in product ratios indicate that the substrate selectivity of Crep1 was modified when compared to positive controls. Yeast harboring either pSG004 (Y150F) or pSG006 (F183W) show a 10-fold and 3-fold decrease, respectively, in 16:2/18:2 product ratio indicating that an 18:1-9c substrate is preferred over a 16:1-9c substrate (Table 4.1). It was also seen that these changes did not affect the amount of 18:2 12-*cis* and 12-*trans* isomers, but instead accumulated less 16:2-9c,12t when compared to positive controls (Figures 3.13 - 3.15). This preference for a longer substrate could be the result of direct modification of the substrate binding channel, affecting substrate positioning in the active site. It can be difficult to understand why a longer-chained substrate should be recognized preferably over a shorter-chained substrate if chain-length recognition is based solely on the position of a pre-existing Δ9 double bond in substrate molecules. Structural changes which influence the size of a substrate binding cavity that accommodate for an 18-carbon long substrate should also be capable of accommodating a shorter 16-carbon long substrate, unless (1) substrate recognition of Crep1 (and likewise other FAD2 enzymes) is influenced by methyl-end recognition, and/or (2) a substrate-binding pocket must not allow excessive movement of the substrate. It should also be noted that the mutations in Crep1, Y150F and F183W, resulted in aromatic amino acids being replaced with comparable aromatic amino acids.

The construct pSG005 (F183L) which replaced the aromatic phenylalanine with a non-aromatic and much smaller leucine resulted in the near ablation of desaturase activity with both 16:1-9c and 18:1-9c substrate (Figures 3.13 - 3.15). Therefore, it is possible that a degree of constriction must exist within the substrate binding cavity for successful desaturation and/or acetylenation to occur.

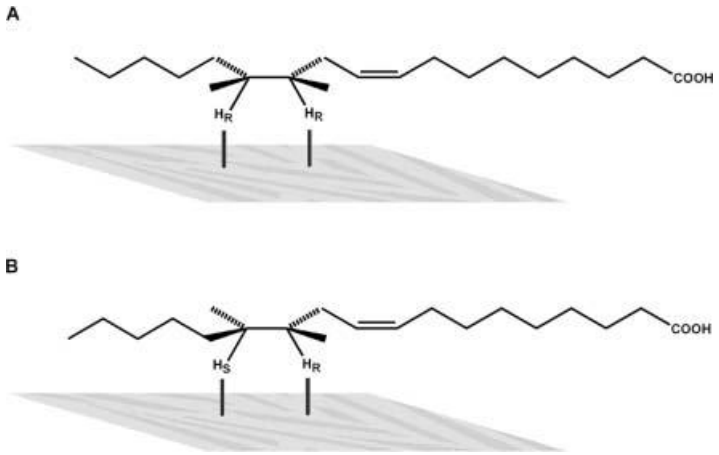

Figure 4.1 Conformations of 18:1-9c Bound to *C. alpina* Δ12 Acetylenase
The *cisoid* conformation in (A) will produce 18:2-9,12c and the *transoid* in (B) will produce 18:2-9c,12t (reproduced from Carlsson *et al.,* 2004).

4.3.4 Accumulation Patterns

As mentioned earlier, the protein expression levels of Crep1 were not determined, and it is therefore not recommended that relative amounts for any fatty acid that may have accumulated be representative of Crep1 activity. The following discussion on the accumulation patterns for Crep1 products is only meant to serve as complementary and supplementary information which adds to the earlier discussion.

Notably, all point mutations resulted in a decrease of 18:1-9c,12a to some degree when compared to positive controls, indicating the importance of each identified amino acid with respect to the acetylenase functionality of Crep1. The greatest decrease in 18:1-9c,12a accumulation was seen in strains harboring pSG004 (Y150F), pSG005 (F183L), and pSG009 (H266Q). Earlier mention of Y150F and F183L associated these mutations with substrate recognition and active site geometry. To reiterate on this matter, the active site and substrate binding cavity appear to be very specific in size to allow for an enzymatic reaction to occur. It is also important to remind the reader that F183L represents a mutation which does not naturally exist in either acetylenases or FAD2s, and the loss of activity caused by F183L is not completely unexpected. H266Q mutants represent a special case in substrate binding (as discussed above; see section 4.3.2) indicating a preference to bind substrate in the *transoid*

conformation. Since Crep1 requires 18:2-9c,12c (possessing a specific *cis* conformation) for the production of 18:1-9c,12a, the inability of the H266Q mutant to bind *cis* stereoisomers would also explain its decreased production of 18:1-9c,12a.

The accumulation of 18:2-9c,12c in transgenic cultures was increased in yeast harboring pSG008 (F259L) and to a lesser degree, pSG010 (V304I) (Table 3.4). The increase in Δ12 *cis* desaturase activity responsible for the increased 18:2-9c,12c accumulation is reflected in the presence of 16:2-9c,12c also detected in these cultures. 16:2-9c,12c was not detected in any other culture including positive controls. The increased desaturase effect of F259L differs from that seen by V304I in two ways. First, a much higher proportion of 18:2 and 16:2 *cis* isomers are produced with mutations at F259L than at V304I, and second, mutations at F259L increased not only the production of the 16:2 and 18:2 *cis* isomers, but of the *trans* isomers as well. This effect was not seen in V304I mutants, which only increased the production of 16:2 and 18:2 *cis* isomers. It appears that F259L amplifies *cis* and *trans* desaturation, whereas V304I accumulated greater amounts of *cis* isomers only.

Some mutations also resulted in decreased 18:2-9c,12c accumulation. The greatest loss was caused by F183L which resulted in 18:2-9c,12c accumulation to levels seen in negative controls, suggesting the ablation of desaturase activity. Other mutants with diminished desaturase activity, including A139S and H306D, appeared to operate at levels comparable to their diminished acetylenase activity.

4 .4 Location of Mutations

These studies indicate that individually replacing either 1 of 4 amino acids affected Crep1 activity the strongest (Table 4.1). Only one of these residues (V304) is directly proximal to the third conserved His-box, whereas the remaining three residues exist in a variety of positions found between the second and third His-boxes. The Y150 residue is located 12 residues past the 2nd His-box; the F259 and H266 residues exists near the interface of the 4th transmembrane domain and cytoplasmic domain (Figure 3.3). These findings are not in agreement with the proposition made by Broun *et al.*, 1998a, who suggested that the amino acids which affect activity the strongest exist near a conserved His-box. Broun *et al.,* 1998a, considered 7 amino acids which differed between desaturases and hydroxylases, and by exchanging as little as 4 of these residues, produced a functional hydroxylase from a

desaturase, and *vice versa*. Only 2 amino acids of the 11 that were detected in this study were also examined by Broun *et al.* The residues found at G97(A) and I317(M) were also described by Broun *et al.*, 1998a, which they label as A104 and M324. During this study, replacing these amino acid residues in Crep1 with those found in analogous positions in FAD2s affected activity only modestly. To help compare the locations of target amino acids in this study with that conducted by Broun *et al.*, 1998a, amino acid targets have been indicated in the Crep1 2-D topological representation (Figure 4.2).

Work conducted by Libisch *et al.*, 2000, which involved the construction of chimeras derived from *Borago officinalis* Δ6 fatty acid desaturase (Boofd6) and Δ8 fatty acid desaturase suggest that the 1st and 2nd predicted transmembrane domains from Boofd6 are involved in the formation of a substrate binding site. Their results were based on the inability of their chimeras to desaturate an 18-carbon long substrate, although were capable of desaturation of a 16-carbon long substrate. Results from this study only highlights the importance of Y150 with regard to substrate specificity, where 18:1-9c could be successfully desaturated, but shorter 16:1-9c could not. My results instead highlight the importance of the last (4th) predicted transmembrane domain with regards to substrate binding, where the H266Q mutant was found to generate increased amounts of *trans* isomers most probably due to substrate binding in a *transoid* conformation. This implies that the last transmembrane domain is of chief importance in stereoselectivity and contributes to a substrate binding channel. Additionally, according to Crep1 topology, this area is predicted to be at the interface of the cytosol and ER membrane, which seems a plausible location for substrate binding to occur.

More recently, Meesapyodsuk *et al.*, 2007, demonstrated that two residues (V or I at 152 and A or V at 206) are important in determining the catalytic activity of CpDes12 and CpDesX desaturases. Residues at position 152 and 206 are analogous to V95 and A149 in Crep1. Although neither of these amino acids was targeted for SDM in my work, they exist next to amino acids G97 and Y150 in Crep1 which were targeted and analyzed in my work. The amino acid G97, which has also been mentioned by Broun *et al.*, 1998a, only yielded modest affects when glycine was exchanged for alanine. Alternatively, Y150 was found to strongly affect Crep1 activity whether regarding relative acetylenase/desaturase activity, substrate selectivity, or stereoselectivity (Table 4.1). It is notable that these locations are likely involved in either the substrate binding site and/or the active site geometry.

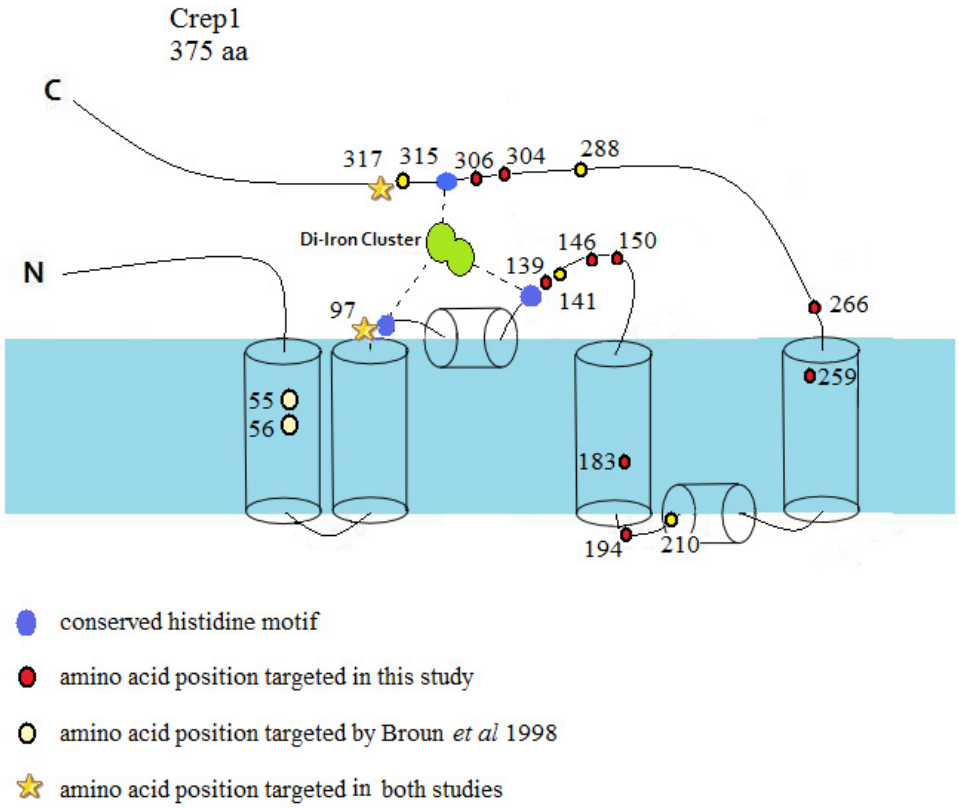

Figure 4.2 Amino Acid Positions Targeted Within Crep1
This diagram compares the amino acid positions which were targeted for site-directed mutagenesis in this study with amino acid positions which were targeted by an earlier study conducted by Broun *et al.*, 1998a. Red circles indicate positions targeted in this work, whereas yellow circles indicate the positions targeted by Broun *et al.*, 1998a. Two positions were exactly the same in both studies and are highlighted by stars. Conserved histidine motifs are represented by blue circles, which are believed to coordinate a diiron cluster (green). Amino acid positions are shown next to the respective circle.

5. CONCLUSIONS

Results from this study have indicated the overall importance of several amino acid locations within the acetylenase Crep1 with regard to chemoselectivity, stereoselectivity, and substrate preference. Of primary importance were 4 amino acid substitutions, F259L, Y150F, H266Q, and V304I, which all contributed different effects on the reaction outcome. F259L affected the desaturase to acetylenase product ratio the greatest, implying its importance in conversion of an acetylenase to an atypical FAD2 capable of producing both *cis* and *trans* isomers, whereas V304I resulted in the conversion of Crep1 into a stereoselective FAD2, where the production of *cis* isomers of 16:2 and 18:2 were increased. The Y150F mutations led to a decrease in acetylenase activity, without any decrease in desaturase activity, also proving an important selector of enzyme activity in Crep1. The H266Q mutation affected substrate selection, seemingly causing an decreased capacity for the enzyme to bind substrate (16:1-9c and/or 18:1-9c) in a *cisoid* conformation, resulting in an increased accumulation of *trans* product. Other amino acid locations also affected relative activity of Crep1, but not greater than mutations at these 4 locations. The locations of these amino acids have been mapped onto a 2-D topological representation of Crep1 and have implicated the cytosolic end of the 4th transmembrane domain in substrate recognition.

Future studies will be required to fully understand the structure/function relationship between acetylenases and desaturases. Further studies regarding the plasticity of Crep1 will be required to better understand its variant activity. Experiments which replace not one, but two or more amino acids within Crep1 would help to enlighten the acetylenase/desaturase relationship. An example of this would be to construct a mutant with both H266Q and F259L mutations and to explore the effects thereafter. Another experiment should investigate mutant constructs in a host other than yeast, since 18:1-9c,12a has been shown to be toxic to the cell (Ford *et al.,* 1986; Croft *et al.,* 1987; Nugteren and Christ-Hazelhof, 1987). Experiments which use *A. thaliana* instead of *S. cerevisiae* have shown to accumulate greater amounts of 18:1-9c,12a (Broun *et al.,* 1998b) and could prove more accurate when determining fatty acid content. Lastly, much of the data produced from this study cannot be validated due to the lack of protein expression validation. Therefore it is important to produce antibodies directed either to Crep1 or to co-express Crep1 with a sequence tag used for antibody detection (*i.e.* histidine tag), which would allow for the confirmation of protein expression.

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