MODIFICATION OF PLANT AND YEAST LIPIDS BY HETEROLOGOUS

EXPRESSION OF PROTIST, ALGAL, AND ANIMAL DESATURASES

By

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A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
School of Molecular Biosciences

December 2006

To the Faculty of Washington State University:

    The members of the Committee appointed to examine the dissertation of REBECCA LYNN OLSEN find it satisfactory and recommend that it be accepted.

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Chair

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AKNOWLEDGEMENTS

MODIFICATION OF PLANT AND YEAST LIPIDS BY HETEROLOGOUS

EXPRESSION OF PROTIST, ALGAL, AND ANIMAL DESATURASES

Abstract

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Modification of lipids by expression of transgenes to alter levels of endogenous fatty acids, and/or to produce novel fatty acids has long been a goal of plant researchers. In this attempt, the identification of desaturases and other fatty acid modification enzymes that can act in heterologous systems was undertaken. *Tetrahymena* species *Tetrahymena pyriformis* and *Tetrahymena thermophila* have three endogenous fatty acid desaturases, a Δ6, a Δ9, and a Δ12. To examine the activity of *Tetrahymena* enzymes, Δ6 and Δ9 desaturases were cloned from a *T. thermophila* cDNA library and modified for expression in heterologous systems. Alignment of these proteins with known desaturases revealed homology seen in conserved domains of these proteins. Desaturation was not seen with the Δ9 desaturase and limited desaturation was seen with the Δ6. However, desaturation was increased 10-fold when the Δ6 desaturase was co-expressed with a cytochrome b_5 from *Arabidopsis thaliana*. Further analysis of the desaturases of *Tetrahymena* involved the cloning of the Δ12 desaturase homologs of both *T. pyriformis* and *T. thermophila* which could account for the Δ14 desaturation of 20:1(11) to 20:2(11,14) seen in *Tetrahymena*. Following the cloning of these desaturase cDNAs, the codons were altered to allow for expression in three heterologous expression systems.

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Desaturation was not seen in any of the systems until the codons were optimized and a small amount of $\Delta 12$ desaturation was seen in yeast. With the overall goal of modification of plant lipids by expression of desaturases two other cDNAs were introduced into *Arabidopsis*, a $\Delta 8$ from *E. gracilis*, and a $\Delta 9$ from *C. elegans*. Expression of the $\Delta 8$ with a seed-specific promoter resulted in the desaturation of 20:1(11) to 20:2(8,11) in *Arabidopsis* seeds. The $\Delta 9$ from *C. elegans* was introduced into *Arabidopsis* seeds plants to reduce the amount of 16:0 saturated fatty acid. Expression of this desaturase with a seed-specific promoter resulted in a reduction of 16:0 from 10% to around 3%. Overall this project highlights the issue that can arise from heterologous expression of desaturases but also the results that can be obtained in alteration of endogenous fatty acids by desaturase expression.

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Dedication

This thesis is dedicated to my family. Thank you.

CHAPTER ONE

GENERAL INRODUCTION

Phospholipids are the major structural constituents of the plasma membrane and the membranes of various eukaryotic organelles. Correct membrane function can be disrupted by changes in fluidity, especially at low temperatures, and organisms that do not regulate their body temperature often have the ability to regulate the fluidity of their membranes. Since membranes with a high ratio of unsaturated fatty acids to saturated fatty acids will remain fluid at reduced temperatures, poikilothermic organisms like *Tetrahymena* species can rapidly adjust the desaturation of the fatty acid components of their membranes in response to lower temperatures (Nozawa et al., 1974; Fukushima et al., 1976; Kasai et al., 1976; Martin et al., 1976; Fukushima et al., 1977; Kitajima and Thompson, 1977; Nozawa and Kasai, 1978).

Lipids have non-structural roles as well. They are important in signaling processes as precursors to such molecules as prostaglandins and leukotrienes in animals, and jasmonic acid in plants. (Anggard and Samuelsson, 1965; Hammarstrom, 1980; Vick and Zimmerman, 1984) In animals, the balance between prostaglandins and leukotrienes regulates the inflammatory response, so that the balance of the precursors for different classes of prostaglandins and leukotrienes is important in cardiovascular health. (Babcock et al., 2000; Connor, 2000; Simopoulos, 2003) In plants, jasmonic acid signaling has a regulatory role in the responses to wounding and to other stress conditions such as high ultraviolet radiation. (Conconi et al., 1996; Howe et al., 1996) Jasmonate also has developmental roles and is critical for anther dehiscence and pollen maturation. (Conconi

et al., 1996; Howe et al., 1996; Stintzi and Browse, 2000) Finally, and importantly in plant oil seeds, lipids act as storage molecules in the form of triacylglycerols (TAGs).

TAGs are comprised of a glycerol backbone where three fatty acid chains are attached.   There is a wide variety in fatty acids found in TAGs. The different fatty acid species are products of fatty acid modification enzymes such as elongases and desaturases. Desaturation enzymes are provided by plants with their specificity of substrate and point of desaturation. Desaturation reactions require molecular oxygen, a pair of reducing electrons, and a desaturating enzyme. Proteins that carry electrons to desaturases include ferrodoxin and cytochrome b5. Interestingly, the desaturases active in the plastid rely on ferrodoxin as an electron donor while the desaturation in the endoplasmic reticulum relies on cytochrome b_5. [1]In seeds and other non-photosynthetic plant tissues, fatty acids are predominantly processed through the eukaryotic pathway where desaturation occurs in the cytosol. Fatty acid substrates for desaturation and elongation are synthesized by fatty acid synthase (FAS) in the plastids, producing a 16-carbon saturated fatty acid bound to acyl carrier protein (16:0-ACP). The majority of 16:0-ACP is elongated to 18:0-ACP by 3-ketoacyl-acyl carrier protein synthase II (KAS II) (Wu et al., 1994). The monounsaturated fatty acid (MUFA) 18:1(9)-ACP is then

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[1] The nomenclature used in the description of fatty acids and the enzymes that act to desaturate or further modify fatty acids can be confusing. When describing fatty acids, it is often easiest to indicate which species is being referred to by the number of carbons it has, the number of double bonds it has, and the location of those double bonds. To do this, they can be expressed in a $X:Y(Z_1,Z_2)$ description where X is the number of carbons, Y is the number of double bonds, and Z_1 and Z_2 are the positions of the double bond from the carboxyl end of the molecule. So an 18-carbon fatty acid with two double bonds at the 9th and 12th carbons from the carboxyl end is 18:2(9,12). Desaturases are also classified by where they form double bonds in the fatty acid. If the enzyme inserts a double bond a specific number of carbons from the carboxyl end of the fatty acid it is a delta (Δ) desaturase. So, a Δ9 desaturase catalyzes the formation of a double bond between the 9th and the 10th carbons from the carboxyl end of the fatty acid. Omega (n or ω) desaturases are described as inserting double bonds a specific distance from the methyl end of the fatty acid. So an ω3 desaturase will generate a double bond between the 3rd and the 4th carbons from the methyl end of the fatty acid.

formed when a Δ9 double bond is introduced into 18:0-ACP by the acyl-ACP desaturase. (Lightner et al., 1994) In seeds about 95% of these fatty acids are then shuttled into the eukaryotic pathway (as 16:0 and 18:1) leaving the plastid and moving into the cytoplasm where they are attached to Coenzyme-A (CoA) and join the acyl-CoA pool.   It is in the cytosol of the seed where further desaturation and elongation can take place.

The only fatty acids that are initially exported from the plastids into the cytosol are 16:0 and 18:1(9). In *Arabidopsis thaliana* seeds, the 18:1(9) can be elongated by the condensing enzyme fatty acid elongation 1 (FAE1) which extends the carbon chain by 2 carbon atoms to form 20:1(11). 20:1(11) makes up 15% of the seed fatty acid. (Kunst et al., 1992) The other abundant fatty acids of seed oil are 18:2(9,12) and 18:3(9,12,15), desaturation products of 18:1 produced by sequential activity of two enzymes, FAD2 (Fatty Acid Desaturase 2) and FAD3. FAD2 is a Δ12 desaturase, (Okuley et al., 1994), and its product, 18:2(9,12), is the substrate for the second enzyme, FAD3, an ω3 desaturase whose product in seed is 18:3(9,12,15) (Browse et al., 1993).

While FAD2 and FAD3 are specific as to substrate and to the site in the fatty acid chain where each introduces its double bond, both are NADH-dependent, membrane-bound desaturases that are found in the endoplasmic reticulum (ER). They also share significant primary structure similarities. One hallmark of all membrane bound desaturases is the presence of conserved amino acids within and adjoining three histidine rich regions. (Avelange-Macherel et al., 1995) In both of these desaturases eight histidine residues are conserved in three domains. These "histidine boxes" of the form $H(X)_{3or4}H$, $H(X)_{2or3}HH$, and $H(X)_{2or3}HH$ are thought to act as an iron-binding motif (Shanklin et al., 1994), stabilizing a di-iron cluster essential for desaturation. The iron

bound to these metalloenzymes is important to the ability of the desaturase to donate the electrons necessary to form a double bond:

$$R_1-CH_2-CH_2-R_2 + O_2 + 2H^+ + 2\ e^- \rightarrow R_1-CH=CH-R_2 + H_2O$$
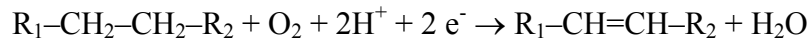
But the di-iron containing desaturase does not act alone, it is dependent on an electron transfer chain that is necessary for the activity of the enzyme. As stated earlier, this group of enzymes found in the cytosol is dependent on transfer of electrons from a cytochrome b_5, which is itself reduced by a NADH-dependent cytochrome b_5 reductase. All members of this electron transport chain are necessary to introduce a double bond into the fatty acids found in the eukaryotic pathway (Strittmatter et al., 1974).

The desaturases acting in the ER can further desaturate 18:1(9) to 18:2(9,12) and 18:3(9,12,15), but 16:0 that is exported into the cytosol is not modified by any endogenous desaturases, so that about 10% of the total seed fatty acids of *Arabidopsis thaliana* are 16:0. This pool of saturated fatty acid is a good target for the possible manipulation of seed lipid composition. The negative effect of saturated fatty acids in human health is well known, and limiting their amounts in common sources of food oils would be beneficial.

Bioengineering of seed oil can have multiple goals, including increasing the amount of oil in seeds, altering the proportions of endogenous fatty acids, and the production of unusual fatty acids that are useful for manufacturing or nutritional purposes (Broun and Somerville, 1997). One goal in the alteration of seed fatty acids is the reduction of saturated fatty-acid composition in seed oil. The experiments described here will demonstrate the effect of expressing FAT-5, a Δ9 desaturase of *C. elegans*, to reduce the percentage of saturated 16-carbon fatty acid in the seeds of this model plant (Chapter

4). Bioengineered plants may also produce fatty acids that are beneficial for human health; the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are good examples. (Horrocks and Yeo, 1999; Connor, 2000) Eicosapentaenoic acid is a 20-carbon fatty acid with five double bonds, 20:5(5,8,11,14,17). The 20:1(11) found in Arabidopsis seeds could be used as a substrate for production of 20:5(5,8,11,14,17), if four additional desaturases were expressed and active in the developing seed. In Chapter 2, I describe the cloning, optimization, and heterologous expression of two desaturases from *Tetrahymena thermophila*, the $\Delta 9$ and the $\Delta 6$. Theses genes were examined for their utility in modifying plant lipids due to their unique enzymatic activities seen in desaturases of *Tetrahymena*. The ability of each of these desaturases to act on fatty acids in yeast was investigated. Co-expression of the $\Delta 6$ with cytochromes of *Arabidopsis thaliana* raised interesting questions about the role of protein-protein interactions in heterologous expression (Chapter Two). I further describe my investigation of the activity of a $\Delta 8$ desaturase isolated from *Euglena gracilis* on the endogenous pool of 20:1; this activity would be useful for producing EPA in *Arabidopsis* seeds (Chapter Four).

Chapter Three describes the cloning, optimization, and heterologous expression of the $\Delta 12$ desaturase homolog from *Tetrahymena pyriformis* and *T. thermophila*; this gene is also likely to be responsible for the unique $\Delta 14$ activity observed in *Tetrahymena* species. My attempts to produce $\Delta 14$ desaturase activity in yeast, nematodes, and plants are described.

CHAPTER TWO

CLONING, OPTIMIZATION, AND EXPRESSION OF THE DELTA 9 AND DELTA 6

DESATURASES OF *TETRAHYMENA THERMOPHILA* AND THE ROLE OF

CYTOCHROME B$_5$ IN DESATURATION

Introduction

Fatty acids play many roles in living cells. They store energy in the form of triglycerides, act as signaling molecules, and have a primary role in membrane structure and function. The ability of organisms to alter the fluidity of organellar and cellular membranes is important in the response of cells to temperature. Membranes will remain fluid at lower temperatures with a high ratio of unsaturated to saturated fatty acids as controlled by the activity of desaturases. One organism in which the response of membrane fatty acid composition to variation in temperature has been studied is the ciliated protist *Tetrahymena thermophila*. (Nozawa et al., 1974; Fukushima et al., 1976; Kasai et al., 1976; Martin et al., 1976; Fukushima et al., 1977; Kitajima and Thompson, 1977; Nozawa and Kasai, 1978) This ciliate, like many other poikilothermic organisms, was shown to rapidly alter the ratio of saturated to unsaturated fatty acids in its membranes in response to temperature. The fatty acid desaturases of *Tetrahymena* have some novel activities such as the ability to desaturate odd chain fatty acids and the unique ability to introduce a Δ14 double bond in a 20 carbon fatty acid substrate. (Lees and Korn, 1966; Cassel et al., 1981) Since the *Tetrahymena* desaturases act on a variety of substrates, they are attractive candidates for use in modification of plant oils. In this chapter the cloning and expression of the Δ6 and Δ9 desaturases of *Tetrahymena* species will be described.

The activity of delta 9 desaturases determines the levels of saturated and unsaturated fatty acids because they introduce the first double bond into a saturated fatty acid. This is a committed step that introduces a cis-double bond between the 9th and 10th carbon in a fatty acid chain. Due to the activity of these desaturases on the saturated substrate 18:0 (stearic acid), these are also known as stearoyl-CoA desaturases (SCDs). All of the members of this Δ9 acyl-CoA desaturase family are membrane bound, with two membrane spanning domains in the protein. These iron-containing proteins also have areas of high amino acid sequence homology at and around three histidine boxes that are essential for enzyme function. (Shanklin et al., 1994) In the nematode *Caenorhabditis elegans*, there are three Δ9 desaturases, FAT-5, FAT-6 and FAT-7. These enzymes have differences in their preferred fatty acid substrate. FAT-5 desaturates 16:0 to 16:1(9), while FAT-6 and FAT-7 desaturate 18:0 to 18:1(9). (Watts and Browse, 2000) *C. elegans* has four other desaturase enzymes, FAT-1, FAT-2, FAT-3 and FAT-4, responsible for ω3, Δ12, Δ6 and Δ5 activities respectively. (Spychalla et al., 1997; Napier et al., 1998; Watts and Browse, 1999; Peyou-Ndi et al., 2000) Examination of the fatty acids of *Tetrahymena* indicates the presence of a Δ9 desaturase that acts on the endogenous saturated fatty acids 16:0 and 18:0. The Δ9 double bond in these monounsaturated fatty acids can then be used as a reference for other enzymes in further desaturation. The polyunsaturated fatty acids (PUFAs) found in *Tetrahymena* implies the presence of enzymes that further desaturate the monounsaturated 18:1.

In *Tetrahymena thermophila*, the PUFAs that are produced demonstrate the activities of both Δ6 and Δ12 desaturases (Figure 1). The monounsaturated fatty acid 18:1(9) is further desaturated to produce 18:2(9,12) and 18:3(6,9,12), the predominant

fatty acids of *Tetrahymena*. Delta 6 desaturases can be grouped into a larger $\Delta 6/\Delta 5$ "front end" desaturase super-family. These are called front end desaturases because they catalyze the formation of a double bond between the $\Delta 9$ double bond and the carboxyl end of the molecule.

The $\Delta 6/\Delta 5$ desaturases can be identified by the characteristics they have in common. Like the $\Delta 9$ desaturases, these enzymes are membrane bound with two membrane spanning regions and have three conserved histidine boxes. Members of the $\Delta 6/\Delta 5$ family also have a conserved cytochrome b_5 domain at their amino terminus. The cytochrome b_5 domain has a conserved HPGG sequence essential for the heme-binding motif of the protein. (Mitchell and Martin, 1995; Sayanova et al., 1999)

Identification of the $\Delta 6$ and $\Delta 9$ desaturases of *Tetrahymena* is made easier by the availability of sequences for the cDNAs. The *T. thermophila* sequence was published by Nozawa et al. (1996), and a search for a *T. thermophila* $\Delta 6$ led to a patent sequence submitted in 2001 (Patent # WO0120000). However, neither of these cDNAs has been heterologously expressed, and therefore they have not been shown to code for the proteins responsible for the desaturase activity.

The codon usage of *Tetrahymena pyriformis* is distinct from that of most eukaryotes. *Tetrahymena* uses a single stop codon, UGA. The codons UAG and UAA, which are stop codons for nearly all eukaryotes, act as glutamine codons. (Helftenbein, 1985; Horowitz and Gorovsky, 1985) Since I wanted to explore the function of the *Tetrahymena* desaturase proteins through expression in *S. cerevisiae*, the stop codons needed to be changed to codons the yeast translation machinery will recognize as glutamine, CAG and CAA. These changes were made using site-directed mutagenesis.

The $\Delta9$ and $\Delta6$ desaturases of *Tetrahymena thermophila* were cloned, modified by correcting stop codons within the open reading frame, and expressed in yeast. The transformation of *ole1* yeast with the $\Delta9$ desaturase of *T. thermophila* failed to rescue the mutant, and no $\Delta9$ desaturase activity was observed. Expression of the $\Delta6$ desaturase in yeast led to a small amount of desaturation. Further investigation of the $\Delta6$ desaturase led to the discovery that co-expression with a cytochrome b_5 from *Arabidopsis thaliana* resulted in a 10-fold increase in desaturation. This finding indicates that the lack of desaturation found in yeast containing the $\Delta6$ desaturase alone was due to inefficiency in electron transfer to the desaturase.

Materials And Methods

Cloning of the delta 6 and delta 9 desaturases

A *Tetrahymena thermophila* cDNA library was requested and received from Dr. Aaron Turkewitz (University of Chicago). The Δ9 desaturase was amplified from the cDNA library by PCR (polymerase chain reaction) using the primers Tthdel9-5'RE and Tthdel9-3'RE (Table 1). The Δ6 desaturase was amplified from the cDNA library using the primers Tthdel6-5'RE and Tthdel6-3'RE (Table 1). Gel extraction was performed on the fragments using the Qiagen Gel Extraction kit, following the published protocol except for an additional wash step of 500μL of 1:5 QG buffer to 2-propanol following the initial spin through of the solubilized gel. Following extraction, the fragment was cloned into pCR-script (Stratagene, La Jolla, CA). Clones were sequenced to verify identity.

Site directed mutagenesis of the desaturases

Mutagenesis of cloned desaturase cDNAs was adapted from procedures previously described (Ho et al., 1989). For the Δ6 desaturase, 11 mutagenic primers were designed, including the 5' terminal primer. The initial round of amplification resulted in 6 fragments. A 224bp fragment was amplified with primers 5'del6mutRE and del6 200R, an 114bp fragment with del6 200F and del6 250R, 387bp with del6 250F and del6 600R, 152bp with del6 600F and del6 700R, 247bp with del6 700F and del6 900R, and a 160bp fragment with del6 900F and del6-3'RE. In the following PCR, approximately equal molar amounts of each fragment along with primers 5'del6mutRE and 3'del6RE were used in amplification with KOD polymerase.   The product that was amplified was the correct size by gel estimation, and was cloned into pCR-script for sequencing.

For the Δ9 desaturase, 6 mutagenic primers were designed in three complementary pairs (Table 1), and the initial round of amplification resulted in the generation of four fragments. These included a 150bp fragment with primers del9-5'RE and del9 100R, 207bp with del9 100F and del9 300R, 207bp with del9 300F and 450R, and a 457bp piece with del9 450F and del9-3'RE. Following the second round of amplification, the PCR product was electrophoresed on an agarose gel and the correct size band was excised. The mutated cDNA was then ligated into pCR-script and sequenced.

Cytochrome b$_5$ proteins of *Arabidopsis*

Clones of three cytochrome b5 cDNAs were received from Dr. Rajesh Kumar and were sequenced to verify identity and accuracy of sequence. Primes were designed for amplification of cytochrome b5 cDNAs to engineer a BamHI site on the 5' end and an XhoI site on the 3' end of the cDNA (Table 1). Fragments amplified by PCR were cloned into pCR-script and vectors were sequenced.

Generation of yeast vectors, and yeast transformation

The mutated Δ6 desaturase was restricted from pCR-script with Acc65I and XhoI and was ligated into the pYES2 vector using NEB T4 ligase, according to the published protocol (New England Biolabs, Ipswitch, MA). The cytochrome b5 cDNAs were restricted from pCR-script with BamHI and XhoI and ligated into the ATCC p414-GPD vector. Using a double-digestion insured the correct orientation of the inserts. Following ligation, 1μL of each of the 10μL ligation reactions was used to transform DH10B electro-competent *E. coli*. (Electromax, Invitrogen, Carlsbad, CA). The plasmid

recovered from the bacteria was then used to transform chemically competent yeast (S. c. EasyComp. Transformation Kit, Invitrogen, Carlsbad, CA). Yeast transformed with the Δ6 desaturase were identified with uracil selection. Yeast transformed with cytochrome cDNAs were identified with tryptophan selection. Yeast were also transformed with the pYES2 empty vector to act as a control.

The mutated Δ9 was restricted out of pCR-script with Acc65I and XhoI, and was then cloned into the p416-GPD vector that had been pre-digested with the same enzymes. (American Type Culture Collection, Manassas, VA). The p416-GPD vector uses a uracil selectable marker and contains the GPD constitutive promoter. The vector construct of the Δ9 in p416-GPD was then sequentially transformed into electro-competent DH10B *E. coli* and then into chemically competent *ole1* yeast. The *ole1* mutant yeast was received from Dr. Charles Martin, and is a knockout of the yeast Δ9 desaturase Ole1p. Transformed yeast were chosen based on uracil selection on plates containing 200μM 18:1(9) and 1%(v:v) DMSO. Yeast were also transformed with the p416-GPD empty vector to act as a control.

Feeding fatty acids to transformed yeast

Yeast cultures were grown in 2x SD media (Clontech, Mountain View, CA). Transformed yeast were fed Δ6 desaturation substrates including 18:1(9), 18:2(9,12), and 18:3(9,12,15) (NuChek Prep, Elysian, MN). These were all obtained as free acids, and were diluted to a working stock of 100mg/mL in DMSO. All cultures were 25ml.

Yeast that was to be analyzed by GC was harvested by centrifugation and were washed with 1% DMSO, 0.1% DMSO and H_2O. Washed yeast was drained of any excess water following the final wash step.

Analysis of fatty acids in yeast

Yeast total fatty acids were derivatized into the fatty acid methyl ester (FAME) by established protocols (Miquel and Browse, 1992). FAMEs were analyzed by gas chromatography on a Supelco SP-2380 capillary column (St. Louis, MO). The derivatized fatty acids were column separated and detected by a flame ionization detector and the identity of the fatty acids were compared to those found in standards run on the same oven profile. Further verification of the Δ6 desaturation products was done by DMOX derivatization. The DMOX protocol was adapted from previously described. The hexane containing the FAME was evaporated, and 9:1 ethanol:benzene was added to the residue, and then evaporated under nitrogen. While still being purged with nitrogen, warmed 2-amino-2-methylpropanol (AMP, at 80ºC) was added to the sample, which was then capped. The sample was baked for 4 hours at 190ºC and then cooled to room temperature. To clean the reaction it was diluted in hexane and then washed twice with H_2O. After excess water was removed, the samples were passed through a Na_2SO_4 drying column and then evaporated until dry. Additional 9:1 ethanol:benzene was added and evaporated. The derivatized samples were then dissolved in a small amount of hexane and separated by gas chromatography followed by detection with a mass spectrometer.

Results

Identification of fatty acid modification enzymes that can be used to bioengineer commercially important fatty acids is one of the major reasons unique desaturases are investigated. These enzymes can be used in the desaturation of plant fatty acids to increase the health benefits of consumed vegetable or other plant oils and in the production of fatty acids for applications in manufacturing or industry. To this end, desaturases from the ciliated protist *Tetrahymena thermophila* were cloned and examined.

Cloning and heterologous expression of the Δ9 desaturase

The sequence of the Δ9 desaturase is available for both the *pyriformis* and *thermophila* species of *Tetrahymena*. (Nakashima et al., 1996; Zhao et al., 1996) A cDNA encoding the *Tetrahymena thermophila* isozyme was amplified from a *T. thermophila* cDNA library (kindly provided by Dr. Aaron Turkewitz) by polymerase chain reaction (PCR). The cDNA was amplified with primers tthdel9-5'RE and tthdel9-3'RE. The 39-mer tthdel9-5'RE included the first 26 nucleotides of the cDNA coding sequence. The 37-mer tthdel9-3'RE included the last 24 nucleotides of the coding sequence including the stop codon TGA (Table 1). These primers also introduced restriction sites at the 5' and 3' ends of the cDNA. The PCR product was cloned into pCR-script and fully sequenced. The coding region of the cDNA was 879bp (including the stop codon), equivalent to the size of the published sequence (Nakashima et al., 1996). The predicted protein product, following translation of the cDNA with a ciliate translation table, was 292aa with a mass of 34.3kD. The ciliate translation table was used

so that the codons TAA and TAG would be treated as glutamine codons and not as stop codons.

To verify that the cloned cDNA aligned to other Δ9 desaturases, the putative *T. thermophila* Δ9 desaturase (Tt del9) was compared to the sequences of known Δ9 proteins (Figure 2). The Δ9 desaturases that were examined were from *Arabidopsis thaliana* (At ADS2), *Caenorhabditis elegans* (Ce FAT-7) and *Drosophila melanogaster* (Dm del9), and were among sequences that were closely related to Tt del9 by a BLAST search of the non-redundant database and confirmed as Δ9 desaturases. (Stukey et al., 1989; Fukuchi-Mizutani et al., 1998; Dallerac et al., 2000; Watts and Browse, 2000) The identity and similarities, respectively, of these protein sequences to that of *T. thermophila* were At – 23.3% and 40.6%, Ce – 34.0% and 48.7%, and for Dm – 32.6% and 47.2%. This family of known Δ9 desaturases has areas of high homology, especially around the three conserved histidine boxes found in the proteins. The putative desaturase isolated from *T. thermophila* does align to these three conserved areas. The first histidine box with the conserved sequence of H(X)$_4$H begins at amino acid 70, the second (H(X)$_2$HH) at 106aa, and the third (H(X)$_3$H) at amino acid 246 (underlined in Figure 2). With this sequence analysis, the cDNA from *T. thermophila* appears to be a Δ9 desaturase and the activity of the cDNA was examined by heterologous expression in yeast.

The yeast *Saccharomyces cerevisiae* makes an almost ideal organism for the heterologous expression of a desaturase. One of the most attractive attributes of this system is that *S. cerevisiae* has only one desaturase, a Δ9, and therefore a relatively simple fatty acid profile (Stukey et al., 1989). To express the Tt del9 cDNA in yeast it was first cloned into a yeast transformation vector. Since Tt del9 is believed to be a Δ9

desaturase, heterologous expression was not done in wild type yeast which already contains a Δ9 desaturase which is encoded by the *OLE1* gene. Instead the vector was transformed into *ole1* yeast which has a mutation in *OLE1* and therefore no endogenous fatty acid desaturase. Without the Δ9 desaturase, *ole1* yeast can only produce saturated fatty acids and do not survive on normal yeast media. *Ole1* yeast requires supplementation with the fatty acid 18:1(9), or another unsaturated fatty acid for survival. If the *T. thermophila* Δ9 desaturase has activity in yeast, the *ole1* mutant phenotype would be rescued, and the yeast could grow without added fatty acid.

To express the Tt del9 in yeast, it first had to be mutagenized to eliminate unwanted stop codons. *Tetrahymena*, like other ciliated protists, only uses one stop codon, TGA, and uses TAA and TAG to code for glutamine. The Δ9 cDNA cloned from the *T. thermophila* cDNA library contained three problem codons, 2 TAA and 1 TAG that were changed to CAA and CAG. The unwanted nucleotides were changed using overlap extension PCR that introduced multiple changes along the cDNA sequence (Figure 3). Primers containing the desired nucleotide changes were designed to regions of the cDNA with unwanted stop codons and used to amplify fragments of the cDNA. The ends of the cDNA fragments were complementary to the primer sequence and therefore contained the desired changes. The primers were also designed so that the fragments would overlap each other and the double stranded DNA in areas of overlap served as primers for extension. The full length cDNA was amplified by including the distal 5' and 3' primers, tthdel9-5'RE and tthdel9-3'RE. After modifying the cDNA, it was cloned into the pCR-script vector and sequenced to confirm the presence of the wanted changes. The cDNA was then sub-cloned into the yeast vector p416-GPD

(American Type Culture Collection, Manassas, VA). This vector contains a uracil selectable marker and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Mutant *ole1* yeast were made competent and transformed with the Δ9 in p416-GPD. Transformed yeast colonies were selected on plates lacking uracil and containing 18:1(9). The ability of the introduced cDNA to complement the *ole1* mutation was tested by transferring transformed yeast to media lacking the supplemented fatty acid. Growth was not seen in media without added 18:1(9), so there was no rescue of the *ole1* mutant phenotype. To see if it was just a lack of desaturation that was failing to rescue the yeast, cultures were also grown in the presence of 18:1(9), and the ability of the transgene to act on the endogenous 16:0 was examined. Cultures were collected and analyzed by GC analysis and spectra did not contain a peak that would correspond to the 16:1 Δ9 desaturation product.

Cloning and heterologous expression of the delta 6 desaturase

In examination of the endogenous fatty acids of *Tetrahymena* species, there appears to be at least three fatty acid desaturases, a Δ6, a Δ9 and a Δ12. To clone the Δ6 desaturase out of *Tetrahymena thermophila*, a search for a candidate with homology to the known members of the Δ5/Δ6 desaturase family led to sequence 1 from the German patent WO0120000. To clone the full length cDNA, primers tthdel6-5'RE and tthdel6-3'RE were used. The 35-mer tthdel6-5'RE included the first 22 nucleotides of the cDNA coding sequence and the 36-mer tthdel63'RE was complementary to the last 23 nucleotides of the cDNA including the TGA stop codon (Table 1). The cDNA was amplified out of a *Tetrahymena thermophila* cDNA library kindly provided by Dr. Aaron Turkewitz, and sequenced. The cDNA from *T. thermophila* was 1059nt from the start

ATG to the TGA stop codon. This cDNA predicts a protein of 352aa with a predicted MW of 41.8 kD. The length of this predicted protein is shorter than many of $\Delta 6$ desaturases from other species, which have an average length of about 440 amino acids. However in amplification of this cDNA out of the cDNA library, no longer cDNA was identified. The prediction of protein sequence again used a translation table for ciliates due to the unusual codon usage of this species.

Before attempting the heterologous expression of the desaturase of *Tetrahymena*, a comparison was made between the *T. thermophila* $\Delta 6$ and members of the same desaturase family previously cloned from other organisms. The predicted protein sequence from *Tetrahymena* was aligned (Figure 4) with those of known $\Delta 6$ desaturases including those from *Borago officinalis* (Bo del6) *Caenorhabditis elegans* (Ce FAT-3) , and *Mortierella alpina* (Ma del6), which were three of the most closely related sequences identified by BLAST search (Sayanova et al., 1997; Napier et al., 1998; Huang et al., 1999). The identity and similarity of these sequences to the *T. thermophila* $\Delta 6$ are Bo – 18.9% and 28.3%, Ce – 21.2% and 29.3%, and to Ma – 19.4% and 29.8%. There are areas of strong conservation in this family of proteins; these are around the three histidine boxes that are found in membrane-bound fatty acid desaturases. This predicted $\Delta 6$ desaturase does have conserved sequence at and around these three histidine boxes. The first box followed the conserved $H(X)_3H$ sequence and started at the 151st amino acid, the second $(H(X)_{2or3}HH)$ began at the 184th amino acid, and the third $(Q(X)_2HH)$ at the 309th amino acid (underlined in Figure 4). The change of the third histidine box from $H(X)_2HH$ to $Q(X)_2HH$ is common to the members of the $\Delta 5/\Delta 6$ "front end" desaturase family. Despite the predicted protein sequence of Tt del6 being shorter than the other

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three Δ6 proteins examined, there is conservation of the residues at and around the histidine boxes.

Along with the presence of these conserved domains, this family of Δ6 desaturases also contains a cytochrome b_5 domain at the amino terminus of the protein. This serves as an electron donor to the desaturase. Like other desaturases, the Δ6 enzymes are dependent on an electron transport chain involving cytochrome b_5 and NADH cytochrome b_5 reductase. Examination of the amino terminus of the Δ6 of *T. thermophila* revealed a cytochrome b_5 domain as expected. This domain includes the highly conserved HPGG sequence starting at residue 42 (starred in Figure 4). The cDNA cloned out of *T. thermophila* had the qualities of a desaturase from this family of proteins as seen by sequence analysis and therefore was examined further.

Having the cDNA believed to encode the Δ6 desaturase of *Tetrahymena*, I attempted to heterologously express the protein in yeast (*Saccharomyces cerevisiae*). Before transformation of yeast could occur, the cDNA was mutagenized to eliminate the unwanted stop codons. In the Δ6 desaturase, there were 8 TAA codons that needed to be changed to CAA, and 2 TAG codons that needed to be changed to CAG. The changes were made using overlap extension PCR as described for the Δ9 desaturase. (Figure 3) The cDNA was sequenced to verify the correct changes were made and that no mistakes were introduced into the sequence. The yeast vector pYES2 was used for expression; this vector contains the GAL1 inducible promoter and a uracil selectable marker.

Yeast has one endogenous fatty acid desaturase, which is a Δ9. With only one desaturase, the fatty acid profile of yeast is very simple, with four main fatty acids, 16:0, 16:1(9), 18:0 and 18:1(9). The activity of heterologously expressed desaturases can be

identified by the appearance of fatty acids not found in wild-type yeast. Yeast will also readily take up fatty acids added to their media which can serve as the substrate for heterologous desaturases. In expression of the *Tetrahymena* Δ6 desaturase, transformed yeast was examined for fatty acids corresponding to Δ6 desaturation. To observe whether transformed yeast had new desaturation activity, cultures were fed fatty acids that were appropriate substrates for desaturation at the Δ6 position. These included 18:1(9), 18:2(9,12) and 18:3(9,12,15). Total yeast lipids were then derivatized to their fatty acid methyl esters (FAMEs) and then analyzed by gas chromatography. With expression of the Δ6 desaturase under the control of the GAL1 promoter a comparison was made between yeast grown in glucose (repression of the GAL1 promoter, and absence of the Δ6) and yeast grown in galactose (induction of the GAL1 promoter and production of the Δ6). In unfed yeast grown in galactose, an additional fatty acid corresponding to the Δ6 desaturation of 18:1(9) to 18:2(6,9) was found in the yeast total lipids. The fed fatty acids 18:2(9,12) and 18:3(9,12,15) were also substrates for Δ6 desaturation. Yeast containing the Δ6 desaturase transgene and grown in galactose did thus appear to contain an active desaturase (Figure 5). Unfed yeast containing the Δ6 desaturase of *Tetrahymena thermophila* desaturated 1.9% of their 18:1(9) to 18:2(6,9) (Figure 5A), yeast fed 18:2(9,12) desaturated 4.7% of the fed fatty acid to 18:3(6,9,12) (Figure 5B) and yeast fed 18:3(9,12,15) desaturated 1.8% of the fed fatty acid to 18:4(6,9,12,15). (Table 3A) The highest amount of desaturation was seen in yeast fed 18:2(9,12), where 4.7% of 18:2 was converted to 18:3(6,9,12). This is very low compared to the desaturation seen in *Tetrahymena* where 50% of 18:2(9,12) is desaturated to 18:3(6,9,12).

Yeast co-expression of Δ6 with *Arabidopsis* cytochrome b₅ proteins

It has been established that the activity of a desaturase depends on an electron transport chain involving NADH cytochrome b_5 reductase and cytochrome b_5 (Dailey and Strittmatter, 1980; Lederer, 1994). There is a cytochrome b_5 domain at the amino end of the Δ6 desaturase from *T. thermophila*, like members of the Δ5/Δ6 family of desaturases. In other members of the Δ5/Δ6 desaturase family, it was shown that this cytochrome b_5 domain was sufficient for electron transfer from the cytochrome b_5 reductase and a second cytochrome b_5 protein was not necessary for desaturation. This model depends on a productive interaction between the endogenous cytochrome b_5 reductase and the cytochrome b_5 domain of the desaturase. If, in yeast, the cytochrome b_5 domain of the *Tetrahymena* Δ6 desaturase acts to receive electrons directly from the yeast cytochrome b_5 reductase, the endogenous cytochrome b_5 of yeast would not be involved. (Figure 9A) However, with the small amount of desaturation seen in yeast it is possible that the cytochrome b_5 domain of the Δ6 desaturase is not able to efficiently receive the electron from the yeast cytochrome b_5 reductase. It is also possible that electron transfer follows the pathway of yeast cytochrome b_5 reductase to yeast cytochrome b_5 to the cytochrome b_5 domain of the Δ6 desaturase. In this case a productive interaction is required between the yeast cytochrome b_5 and the cytochrome b_5 domain of the desaturase (Figure 9B). There is some electron transport occurring because Δ6 desaturation is observed, just a limited amount. It is known by looking at the fatty acid profile of *Tetrahymena* species, that the Δ6 desaturase in the organism is much more efficient than 5% desaturation of substrate to product. One of the most abundant fatty acid in *Tetrahymena* is 18:3(6,9,12), which requires the Δ6 double bond.

To see if desaturation could be improved by co-expression with a cytochrome b5 protein, the Δ6 desaturase of *Tetrahymena thermophila* was co-transformed into yeast with an *Arabidopsis* putative cytochrome b_5 cDNA, one of three used. *Arabidopsis* cytochrome b_5 cDNAs were used since modification of seed oil lipids is the final goal of this project, and *Arabidopsis* is the model plant we work with. If this desaturase was expressed in *Arabidopsis*, these are the cytochrome b_5 proteins that would be available. The *Arabidopsis* proteins that were chosen to work with were ATB5-A (At5g56530), ATB5-B (At5g48810), and B5 #4 (At2g32720). The cDNAs for each of these cytochrome b_5s were individually introduced into the p417 vector with the GDP constitutive promoter and a histidine selectable marker. Three sets of double transformants were isolated by plating on media selective for uracil and histidine, Tt del6 with ATB5-A, Tt del6 with ATB5-B and Tt del6 with B5#4. Single transformants with each of the cytochrome b_5s alone were also generated, as controls. Cultures were then treated as before for the Δ6 transformed yeast and were screened for Δ6 desaturation.

The production of Δ6 desaturation products was increased in all of the yeast strains that contained both the Δ6 and an Arabidopsis cytochrome b_5, but the most drastic result was when the desaturase was co-expressed with ATB5-B. The new desaturation levels were 10-fold those of the desaturase without the cytb$_5$. Yeast that were grown in galactose without fatty acids desaturated 21.6% of their 18:1(9) to 18:2(6,9) (Figure 6), yeast fed 18:2(9,12) and grown in galactose desaturated 44.4% of the fed fatty acid to 18:3(6,9,12) (Figure 7) and yeast fed 18:3(9,12,15) and grown in galactose desaturated 32.0% of the substrate to 18:4(6,9,12,15) (Figure 8, Table 2B). Also, unlike yeast containing the Δ6 alone, the yeast with the cytochrome b5 and the desaturase were able to

desaturate 16:1(9) to 16:2(6,9) with 7.3% desaturation (Figure 6). The identities of all

the fatty acid products were verified by DMOX derivatization of the FAMEs and analysis

on a gas chromatograph coupled with a mass spectrometer.

Discussion

Saccharomyces cerevisiae has only one fatty acid desaturase, a Δ9, and therefore has a relatively simple fatty acid profile with 16:0, 16:1(9), 18:0 and 18:1(9) as greater that 95% of the fatty acids. Because of this lack of polyunsaturated fatty acids, yeast are commonly used for the heterologous expression of desaturases for verification of activity and identification of substrate preference. However, heterologous expression of transgenes is not always successful. Expression of the Δ9 and Δ6 desaturases of *Tetrahymena thermophila* illustrates the difficulties that can occur. The cDNA thought to encode the Δ9 desaturase of *T. thermophila* was cloned from a cDNA library and transformed into mutant yeast that did not possess the endogenous Δ9 fatty acid desaturase, Ole1p. The inability of the Tt del9 transgene to complement the mutant phenotype of *ole1* yeast could have many causes including improper membrane localization of the desaturase or substrate unavailability. If components needed for activity of the desaturase, such as an efficient electron transport chain, were compromised then this could also result in lack of desaturation. Unlike the Δ9 desaturase of yeast, Ole1p, or the Δ6 desaturase of *Tetrahymena*, the Δ9 desaturase of *Tetrahymena thermophila* does not have a cytochrome b_5 domain. Therefore, the desaturase is dependent on electron transfer from the endogenous cytochrome b_5 of yeast. If the desaturase cannot effectively receive electrons from the yeast cytochrome b_5, it would not be able to catalyze the introduction of a double bond. This question might be answered if co-expression of the Δ9 desaturase and an *Arabidopsis* cytochrome b_5 resulted in desaturation and rescue of the *ole1* phenotype.

The *Tetrahymena thermophila* Δ6 was expressed in yeast under the control of the GAL1 promoter. When yeast were grown in galactose, a small peak corresponding to Δ6 desaturation was observed. However, the activity of the desaturase was very limited as compared to other Δ6 desaturases expressed in yeast and compared to the endogenous activity in *Tetrahymena*. When the Δ6 from *C. elegans* (FAT-3) was expressed in yeast, 14% of fed 18:2(9,12) was desaturated to 18:3(6,9,12), and when the Δ6 from *B. officinalis* was transformed into yeast 44.7% of fed 18:2(9,12) was desaturated to 18:3(6,9,12). (Watts and Browse, 1999; Sayanova et al., 2001) In *Tetrahymena*, the polyunsaturated 18-carbon fatty acids, 18:2(9,12) and 18:3(6,9,12), are present in approximately equal amounts and are the most abundant fatty acids in this organism. (Figure 8A) This would indicate that about 50% of the available Δ6 substrate (18:2(9,12)) is desaturated to 18:3, as compared to the 4.7% desaturation seen here. Because there is some activity, it appears that the desaturase is correctly localized in the membranes of the yeast and that there is availability of substrate for desaturation.

Electrons are necessary for desaturases to catalyze the formation of a double bond. This group of membrane bound desaturases gets their electrons from a donor, cytochrome b_5, which in turn gets its electrons from a cytochrome b_5 reductase. This forms the electron transport chain necessary for desaturation. In an attempt to increase the amount of Δ6 desaturation, the *Tetrahymena* desaturase was co-expressed with a member of the desaturase electron transport chain, cytochrome b_5. The cDNAs chosen were those of three *Arabidopsis* cytochrome b_5s, ATB5-A, ATB5-B, and B5#4. When the ATB5-B cytochrome b_5 was co-expressed with the *Tetrahymena thermophila* Δ6, the desaturation was increased 10-fold. This result raises questions about the electron

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transport that is occurring in yeast. There are three cytochrome b_5 proteins that need to be considered in yeast co-transformed with the *Tetrahymena* Δ6 and the cytochrome b_5 of *Arabidopsis*. There is the endogenous cytochrome b_5 of yeast, the *Arabidopsis* cytochrome b_5 and the cytochrome b_5 domain of the Δ6 desaturase. In yeast transformed with the Δ6 desaturase, there are two main possibilities for the electron transport that is occurring. The first possibility is that the desaturase should be receiving electrons directly from the yeast cytochrome b_5 reductase, but that there is a problem in the necessary protein-protein interaction. (Figure 9A) A second possibility is that the yeast cytochrome b_5 reductase is giving its electrons to the yeast cytochrome b_5, but that this is not able to pass the electrons to the cytochrome b_5 domain of the Δ6 desaturase. (Figure 9B) It has been shown previously with Δ6 desaturases containing a cytochrome b_5 domain that the presence of another cytochrome b_5 is not necessary for desaturation. However, when another cytochrome b_5 is present, desaturation is increased.(Michinaka et al., 2001; Guillou et al., 2004) With this data, it is likely that both pathways (Figure 9 A + B) are active in yeast. So with the cytochrome b_5 domain of the *Tetrahymena* Δ6 desaturase, and the cytochrome b_5 of yeast, why is there so little desaturation seen in transformed yeast? One possibility is that the limited desaturation seen in yeast expressing the *Tetrahymena* Δ6 desaturase alone was due to a lack of cytochrome b_5. In this scenario the increase in Δ6 desaturation seen with the co-expression of the *Arabidopsis* cytochrome b_5 proteins was be due not to specific interactions with the *Arabidopsis* cytochrome b_5 but to the presence of additional cytochrome b_5 proteins. This possibility will be addressed by over-expressing the *S. cerevisia*e cytochrome b_5 in yeast transformed with the *T. thermophila* Δ6 desaturase cDNA. However, it appears

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that the lack of desaturation seen in yeast containing the is due to the inability of the

cytochrome b_5 domain of the protein to properly interact with either the cytochrome b_5 or

the cytochrome b_5 reductase of yeast. It was to address this issue that the *Tetrahymena*

$\Delta 6$ desaturase and the *Arabidopsis thaliana* cytochrome b_5 were co-expressed in yeast

With another cytochrome b_5 protein there are now two new possible scenarios for

the electron transport chain. In the more likely scenario the cytochrome b_5 reductase of

yeast is able to give electrons to the cytochrome b_5 of *Arabidopsis* and this cytochrome b_5

can then interact with the cytochrome b_5 domain of the $\Delta 6$ protein and desaturation can

occur. (Figure 9 C) There is also the possibility that all three cytochrome b_5 proteins are

involved with the cytochrome b_5 reductase giving electrons to the yeast cytochrome b_5,

and that the electrons are then give to the cytochrome b5 domain of the desaturase via the

Arabidopsis cytochrome b_5. (Figure 9 D) In all these scenarios, the cytochrome b_5

domain of the $\Delta 6$ desaturase is involved. In previous studies of the $\Delta 6$ desaturases of rat

and borage, a mutation of the HPGG domain of the desaturase, which is essential for

cytochrome b_5 activity, resulted in the complete loss of function for the desaturase. This

loss of function was seen even in the presence of endogenous cytochrome b_5 proteins and

with the over expression of additional cytochrome b_5 proteins. For this reason, I believe

it is unlikely that the *Tetrahymena* desaturase is receiving electrons from the *Arabidopsis*

cytochrome b_5 and bypassing the cytochrome b_5 domain. However, this possibility will

be addressed by mutation of the histidine of the conserved HPGG domain of the

Tetrahymena $\Delta 6$ cytochrome b_5 domain.

Heterologous expression of proteins does not always result in the same levels of

activity in the expression system as seen in the host organism. In the case of the $\Delta 6$

desaturase, the levels of desaturation were drastically reduced until a second "helper"

protein was co-expressed. This highlights an issue with heterologous protein expression.

The host system cannot be recreated identically, but if essential proteins that are

necessary for activity in the host system can be identified and co-expressed in the

expression system, it is possible to more closely recreate activities seen endogenously.

Table 1. Primers used for the amplification of delta 6 and delta 9 cDNAs from a *T. thermophila* cDNA library, and the mutagenesis
Of the cDNAs to remove unwanted stop codons.

	Primer name	Mutagenesis site	Primer sequence
Delta6	tthdel6-5'RE		GGTACCGGATCCATGGGAGTTGATAAGACTTAAGA
T. th	del6 200F	T169C, T208C	GAGAAGCAAGATTTGACTGAATATTTCAGAACACTCCATTCTAAGCAGGC
	del6 200R	T169C, T208C	GCCTGCTTAGAATGGAGTGTTCTGAAATATTCAGTCAAATCTTGCTTCTC
	del6 250F	T253C	GGCGCAAAACAAGAGGAGACTGAATCTTCAAAG
	del6 250R	T253C	CTTTGAAGATTCAGTCTCCTCTTGTTTTGCGCC
	del6 600F	T601C, T622C	CGAAGATATCCAACACGATTACAAATTGTGGCAATTCCCC
	del6 600R	T601C, T622C	GGGGAATTGCCACAATTTGTAATCGTGTTGGATATCTTCG
	del6 700F	T727C	GGGTATTATTATTCAACCAAAACTTCTATATCG
	del6 700R	T727C	CGATATAGAAGTTTTGGTTGAATAATAATACCC
	del6 900F	T919C, T925C, T949C	GGTATGCAATATCAGACTGAACATCACTTTTTCCCACAAATTCC
	del6 900R	T919C, T925C ,T949C	GGAATTTGTGGGAAAAAGTGATGTTCAGTCTGATATTGCATACC
	tthdel6-3'RE		CTCGAGCGGCCGCTCAAAGGTGAGATTTTTCAAAAA
Delta 9	tthdel9-5'RE		GGTACCGGATCCATGGGAGTATACTCTAAAGGTGAATTC
T. th	thdel9 150F	T121C	GGTCTTATCTACTGGCTTTTACAAGAAAAGGAATTGTTC
	thdel9 150R	T121C	GAACAATTCCTTTTCTTGTAAAAGCCAGTAGATAAGACC
	thdel9 300F	T286C	CTTTAACAGCATGGCCTTCCAAGGTTCTATTTTCCACTGG
	thdel9 300R	T286C	CCAGTGGAAAATAGAACCTTGGAAGGCCATGCTGTTAAAG
	thdel9 450F	T454C	GGTGAAAAATTGAATATTCAGGATCTTAAGGATGACCC
	thdel9 450R	T454C	GGGTCATCCTTAAGATCCTGAATATTCAATTTTTCACC
	tthdel9-3'RE		CTCGAGCGGCCGCTCAAGATTCGTTTTTCTTAATTCC

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Table 2. Desaturation in yeast transformed with the delta 6 desaturase of *Tetrahymena thermophila* or co-transformed with the delta 6 desaturase and the cytochrome b5 CBR-B of *Arabidopsis thaliana.*

Fatty Acid	Ttdel6 in pYES2 Glucose grown		Ttdel6 in pYES2 Galactose Induced	
	Percent Substrate	Desaturation	Percent Substrate	Desaturation
endogenous FAs				
16:1 Δ 9	36.7	0	36.0	0
18:1 Δ 9	32.7	0	34.1	1.9
fed FAs				
18:2 Δ 9,12	16.3	0	18.4	4.7
18:3 Δ 9,12,15	19.3	0	24.0	1.8

Fatty Acid	Ttdel6 in pYES2/CBR-B in p416-GPD Glucose grown		Ttdel6 in pYES2/CBR-B in p416-GPD Galactose Induced	
	Percent Substrate	Desaturation	Percent Substrate	Desaturation
endogenous FAs				
16:1 Δ 9	31.7	0	33.2	7.3
18:1 Δ 9	27.5	0	19.9	21.6
fed FAs				
18:2 Δ 9,12	30.0	0	13.9	44.4
18:3 Δ 9,12,15	31.5	0	22.8	32.0
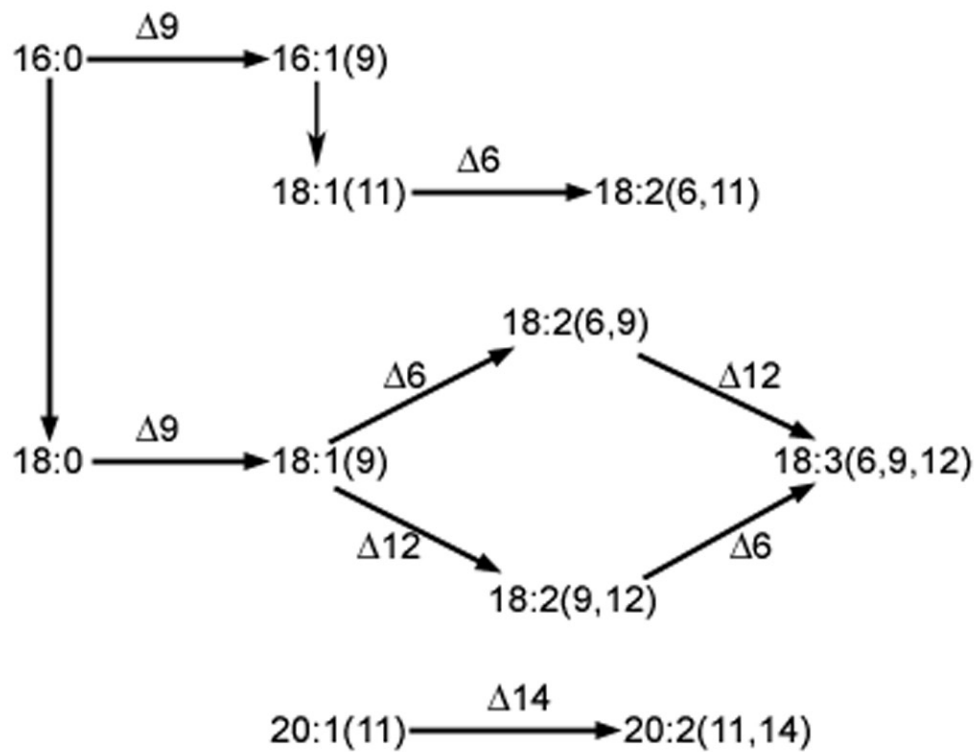
Figure 1. Pathway of desaturation and elongation of *Tetrahymena* species based on the fatty acids of *Tetrahymena* and feeding studies. The 16- and 18-carbon fatty acids are endogenous to *Tetrahymena*. Action of the delta 14 desaturase is seen only in the presence of the fed fatty acid, 20:1.

```
              1                                                               80
At ADS2   (1) ------------------------MSVTSTVEENHQ----------KNPSTPAAVEEKKKRRWVFWDRRWRRLDYVKF
Ce FAT-7  (1) -------------------MTVKTRASIAKKIEKDG----LDSQYLFMDPNEVLQVQEESKKIPYKMEIVWRNVALFAA
Dm del9   (1) MPPNAQAGAQSISDSLIAAASAAADAGQSPTKLQEDSTGVLFECDVETTDGGLVKDITVMKKAEKRLLKLVWRNIIAFGY
Tt del 9  (1) ------------------------------------------------------MGVYSKGEFKVMGYELKKEYCIWLGY

              81                                                              160
At ADS2  (45) SASFTVHSLALLAPFYFT--WSALWVTFLFYTIGGLGITVSYHRNLAHRSFKVPKWLEYLLAYCALLAIQGDPIDWVSTH
Ce FAT-7 (57) LHVAAAIGLYELVFHAK---WQTAVFSFALYVFSGFGITAGAHRLWSHKSYKATTPMRIFLMLLNNIALQNDIIEWARDH
Dm del9  (81) LHLAALYGAYLMVTSAK---WQTCILAYFLYVISGLGITAGAHRLWAHRSYKAKWPLRVILVIFNTIAFQDAAYHWARDH
Tt del 9 (27) MHILAVAGLIYWLLQEKELFTKSLYMFLIFHLMGGLGITAGSHRLWAHRSYTAKTPYRILVMLFNSMAFQGSIFHWSRDH

              161                                                             240
At ADS2 (123) RYHHQFTDSERDPHSPKEGFWFSHLLWIYDSAY-LVSKCGRRANVEDLKR------QWFYRFLQKTVLFHILGLGFFLFY
Ce FAT-7(134) RCHHKWTDTDADPHNTTRGEFFTHMGWLLVRKHPQVKEHGGKLDLSDLFSDPVLVFQRKHYFPLVILFCFILPTIIPVYF
Dm del9 (158) RVHHKYSETDADPHNATRGEFFSHVGWLLCKKHPEVKAKGKGVDLSDLRADPILMFQKKYYMILMPIACFIIPTVVPMYA
Tt del 9(107) RLHHKFSDTDIDPHSMNKGEFFAHVGWLLVKKSPELKKEGEKLNIQDLKDDPVVMFQKKHYFLISTLVCFVIPTVIMQYT

              241                                                             320
At ADS2 (196) LGGMSFVTWGMG--VGAALEVHVTCLINSLCHIWGTRTWKTNDTSRNVWWLSVFSFGESWHNHHAFESSARQGLE-WWQ
Ce FAT-7(214) WKETAFIAFYVAGTFRYCFTLHALWCINSAAHYFGWKPYDTSVSAVENVFTTVVAVGEGGHNFHHTFPQDYRASEY-SLI
Dm del9 (238) WGESFMNAWFVATMFRWCFILNVLWLVNSAAHKFGGRPYDKFINPSENISVAILAFGEGWHNYHHVFPWDYKTAEFGKYS
Tt del 9(187) IGGGFIYNFLMS-CFRYVFMLHTLWCVNSVCHMFGSRPYNESILPTENLFVSIIALGEGWHNWHHEYPRDWRAAENKWTK

              321                                                     386
At ADS2 (273) IDISWYIVRFFEIIGLATDVKVPTEAQRRRMAIVR-----------------------------
Ce FAT-7(293) YNWTRVLIDTAAVLCLVYDRKTIADEFISRQVANHGS--------------EESRKKSIM------
Dm del9 (318) LNFTTAFIDFFAKIGWAYDLKTVSTDIIKKRVKRTGDGTHATWGWGDVDQPKEEIEDAVITHKKSE
Tt del 9(266) INFTSGFIKFFEFLGQCDTNGIKKNES--------------------------------------
```

Figure 2. Delta 9 desaturase from *T. thermophila* is homologous to *Arabidopsis thaliana* ADS2, *Caenorhabditis elegans* FAT-7 and *Drosophila melanogaster* delta9. Areas of high conservation are found around the three histidine boxes, underlined. Conserved sequence for the first histidine box is HXXXXH, for the second HXXHH and for the third HXXHH. Conserved residues are boxed in grey.

A. *Tetrahymena* cDNA with unwanted stop codons (underlined)

TATAAG   ATTTAGG

B. Primers designed for site directed mutagenesis including nucleotide change (in red)

ATGTTC

TACAAG   TAAGTCC

ATTCAGG

C. Fragments amplified including nucleotide changes at ends are amplified with far
5' and 3' primers to generate full length cDNA

ATGTTC

TACAAG   TAAGTCC

ATTCAGG

D. Full length cDNA with desired changes

TACAAG   ATTCAGG

Figure 3. Site directed mutagenesis using overlap extension PCR. The cDNA cloned from Tetrahymena included unwanted stop codons (A) that needed to be changed for heterologous expression. Primers were designed to include desired changes (B) and used to amplify fragments containing the new, non-stop, codons (C). The full length cDNA (D) was then amplified using the distal 5' and 3' primers.

```
                    1                                                                    80
Bo del6    (1)   ---------MAAQIKKYITSDELKNHDKPGDLWISIQGKAYDVSDWVK--DHPGGS--FPLKSLAGQEVLDAFVAFHP--
Ce FAT-3   (1)   ---------------------MVVDKNASGLRMKVDGKWLYLSEELVK--KHPGG---AVIEQYRNSDATHIEHAFHEGS
Ma del6    (1)   MAAAPSVRTFTRAEILNAEALNEGKKDAEAPFLMIIDNKVYDVREFVP--DHPGG---SVILTHVGKDGTDVEDTFEP--
Tt del6    (1)   ----------MGVDKTQEEIVLENKPELLNEYKFIYKDTEYDCTEYAKSNKHPGGLNFLNLFIDEKQDLIEYFRTLHS--
                                                                               ****
                    81                                                                   160
Bo del6    (66)  --------------------ASTWKNLDKFFTGYYLKDYSVSE---VSKDYRKLVFEFSKMGLYDKKGHIMFATLCFIA
Ce FAT-3   (55)  SQAYKQLDLLKKHGEHDEFLEKQLEKRLDKVDINVSAYDVSVAQEKKMVESFEKLRQKLHDDGLMKANETYFLFKAISTL
Ma del6    (74)  -------------------EAAWETLANFYVGDIDESDRAIKN-DDFAAEVRKLRTLFQSLGYYDSSKAYYAFKVSFNL
Tt del6    (69)  --------------------KQALKILKSFPKTGAKQEETESS-----KRFSILKKKLKHLFEPNWPIEIGLFLTTFTL
                    161                                                                  240
Bo del6    (122) MLFAMSVYGVLFCEG--VLVHLFSGCLMGFLWIQSGWIGHDAGHYMVVSDSRLNKFMGIFAANCLSCISIGWWKWNHNAH
Ce FAT-3   (135) SIMAFAFYLQYLG-----WY-ITSACLLALAWQQFGWLTHEEFCQQPTKNRPLNDTISLFFGNFLQCFSRDWWKDKHNTH
Ma del6    (133) CIWGLSTFIVAKRGQTSTLANELSAALGLFWQQRGWLAHDFLHQVFQDRFWGDLFGAFLGGDCQCFSSSWWKDKHNTH
Tt del6    (123) FVTGCLTQKWYFS----------IP-LLVLMQIISGWIGHSMNHNRNP----ILRKFALVYAPLCGCFSNKWWGRKHNQH
                    241                                                                  320
Bo del6    (200) HIACNSLEYDPDLQYIPFLVVSSKFFGSLTSHFYEKRLTFDSLSRFFVSYQHWTFYPIMCAARLNMYVQSLIMLLTKRNV
Ce FAT-3   (209) HAATNVIDHDGDIDLAPLFAFIPGDLCKYKASFEKAILKIVP-------YQHLYFTAMLPMLRFSWTGQSVQWVFKENQM
Ma del6    (213) HAAPNVHGEDPDIDTHPLLTWSEHALEMFSDVPDEELTRMWSR--FMVLNQTWFYFPILSFARLSWCLQSILFVLPNGQA
Tt del6    (188) HMFTNNILKDEDIQHD-----------------------------YKLWQFPFLFLKWKLDSILASYYEFEG----
                    321                                                                  400
Bo del6    (280) SYRAHELLG--------CLVFSIWYPLLVSCLPNWGE--RIMFVIASLSVTGMQQVQFSLNHFSSSVYVGK-PKGNNWFE
Ce FAT-3   (282) EYKVYQRN--AFWEQATIVGHWAWVFYQLFLLPTWPL-RVAYFIISQMGGGLLIAHVVTFNHNSVDKYPANSRILNNFAA
Ma del6    (291) HKPSGARVPISLVEQLSLAMHWTWYLATMFLFIKDPVNMMVYFLVSQAVCGNLLAIVFSLNHNGMPVISKEEAVDMDFFT
Tt del6    (231) ----------------------IFLALHWVLLFNQN--FYIVILSELIAGFFSASILVGNHENEMKFERR--ITLPFFE
                    401                                                                  480
Bo del6    (349) KQTDGTLDISCP-PWMDWFHGGLQFQIEHHLFPKMPRCNLRKISPYVIELCKHNLPYNYASFSKANEMTLRTLRNTALQ
Ce FAT-3   (359) LQILTTRNMTPS-PFIDWLWGCLNYQIEHHLFPTMPRCNLNACVKYVKEWCKENNLPYLVDDYFDGYAMNLQQLKNMAEH
Ma del6    (371) KQIITGRDVHPG-LFANWFTGCLNYQIEHHLFPSMPRHNFSKIQPAVETLCKYGVRYHTTGMIEGTAEVFSRLNEVSKA
Tt del6    (284) HQIAASRNYAFHDIFSLLIMGCMQYQTEHHFPQIPFYRLPKARVIIAEELKKWNLKIHEGPIFEKSHL-----------
                    481         501
Bo del6    (428) ARDITKPLPKNLVWEALHTHG
Ce FAT-3   (438) IQAKAA---------------
Ma del6    (450) ASKMGKAQ-------------
Tt del6    (353) --------------------
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Figure 4. Sequence of the delta 6 desaturase of *Tetrahymena thermophila* aligns with known delta 6 desaturases. The Tt del6 is homologous to delta 6 desaturases from Borage officinalis (Bo del6), C. elegans (Ce FAT-3), and Mortierella alpine (Ma del6), especially in regions around the three histidine boxes (underlined), and the conserved cytochrome b_5 HPGG sequence (starred). Conserved residues are boxed in grey.
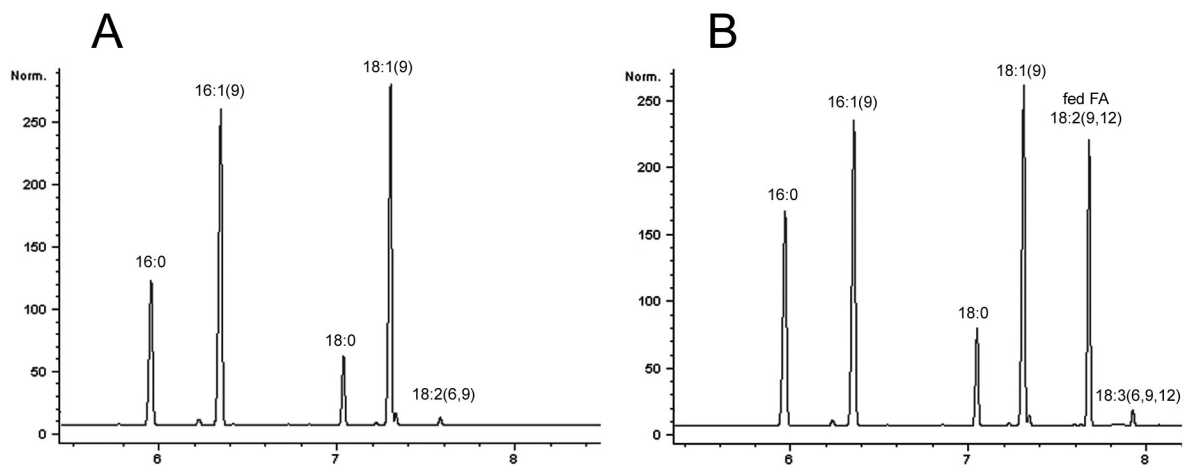
Figure 5. Activity of the *T. thermophila* delta 6 desaturase in yeast. Fatty acids of yeast transformed with the delta 6 desaturase homolog of *T. thermophila* in pYES2 and grown in media containing galactose were derivatized to FAMES and run on the GC to look for delta 6 desaturation peaks. Yeast not fed fatty acids (A) and yeast fed 18:2(9,12) (B) had peaks corresponding to the delta 6 desaturase products 18:2(6,9) and 18:3(6,9,12), respectively.
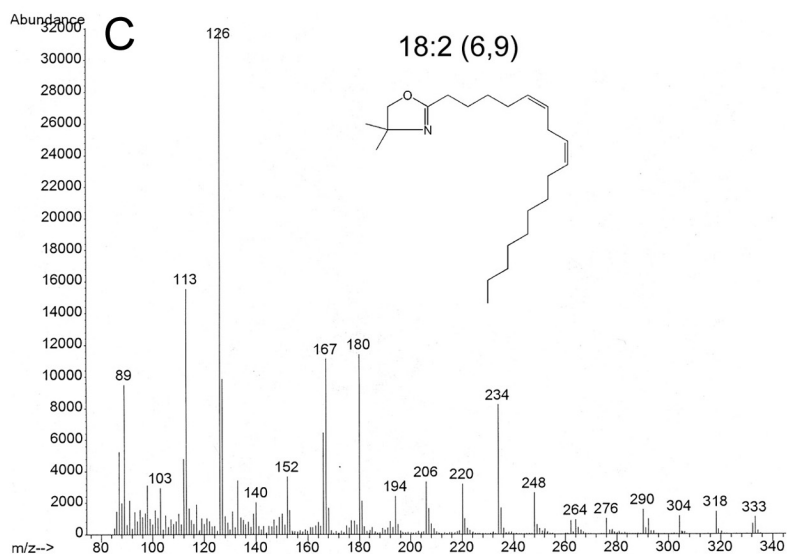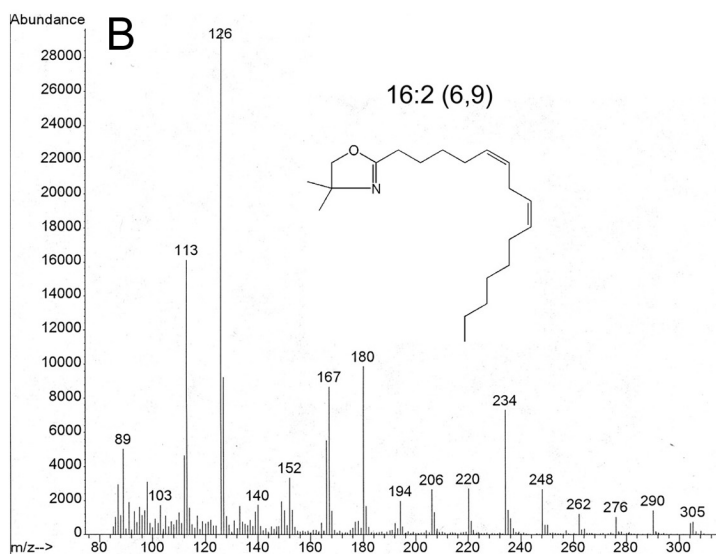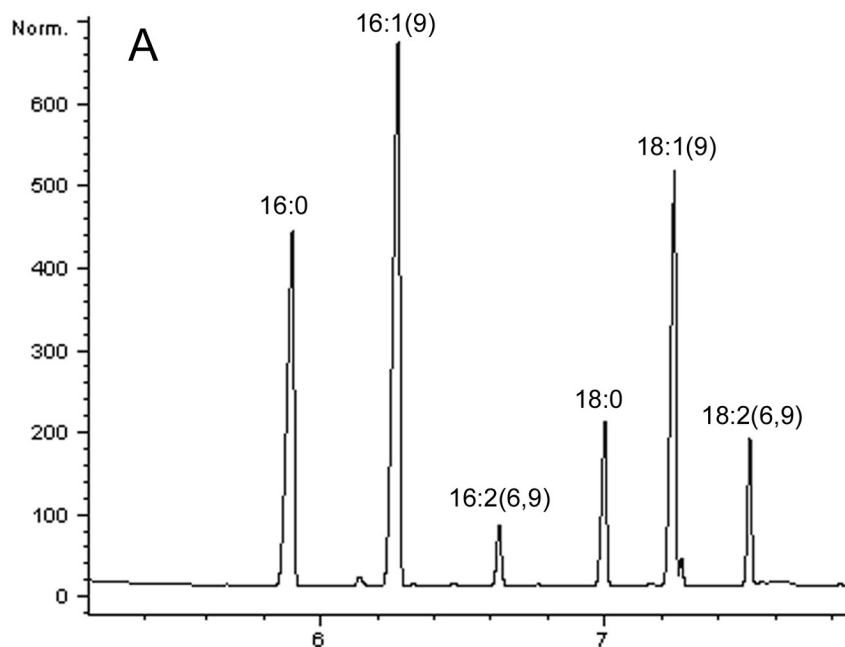
Figure 6. Identification of delta6 desaturation of endogenous yeast fatty acids. Yeast transformed with the delta6 desaturase of *Tetrahymena thermophila* in pYES2 and the *Arabidopsis thaliana* cytochromeb5, AtCYB5-B, were grown with galactose induction. Fatty acid methyl esters of the transformed yeast contain the endogenous saturated and monounsaturated fatty acids of yeast and the delta6 desaturation products 16:2(6,9) and 18:2(6,9) (A). Verification of 16:2(6,9) by GC-MS of the DMOX derivative (B). Verification of the DMOX derivative of 18:2(6,9) (C).
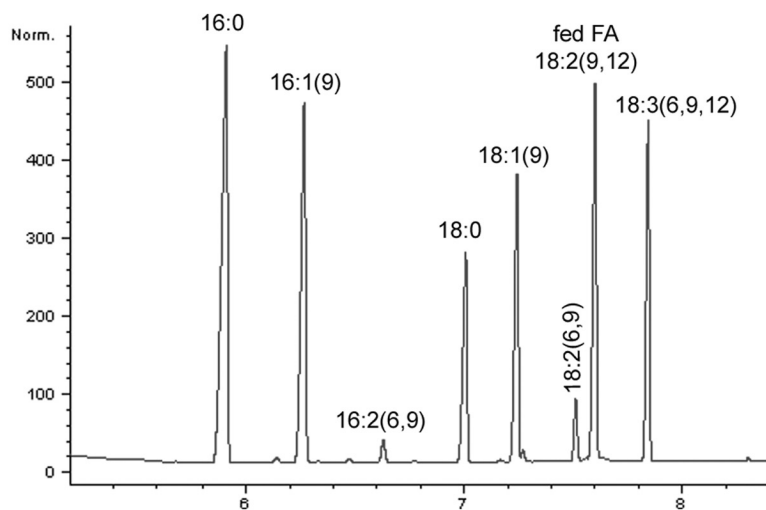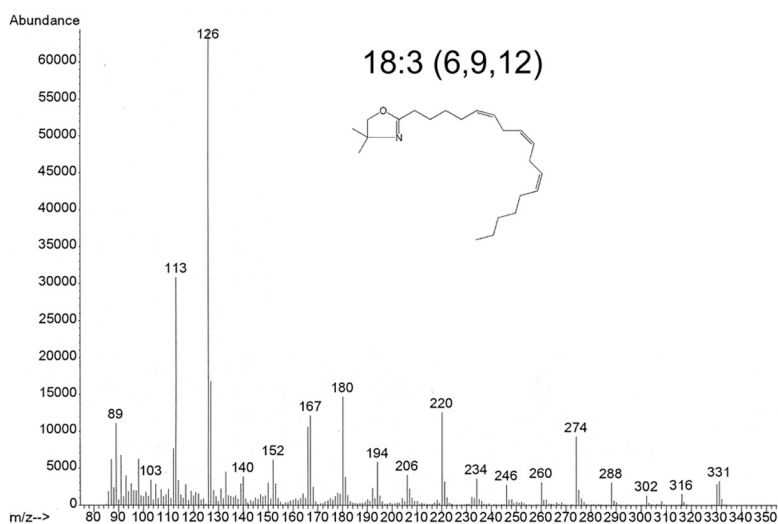
Figure 7. Desaturation of 18:2(9,12) to 18:3(6,9,12) in yeast. Yeast transformed with pYES2 containing the delta6 of *T. thermophila* and AtCYB5-B in p414-GPD were fed 18:2(9,12) and induced by galactose. (A) GC trace of fatty acid methyl ester derivatives reveals the delta6 desaturation products 16:2, 18:2(6,9) and 18:3(6,9,12). 16:0, 16:1, 18:0 and 18:1 are endogenoud yeast fatty acids. 18:2(9,12) is the fed delta6 fatty acid substrate. (B) GC-MS of the DMOX derivative verifying the identity of 18:3(6,9,12)
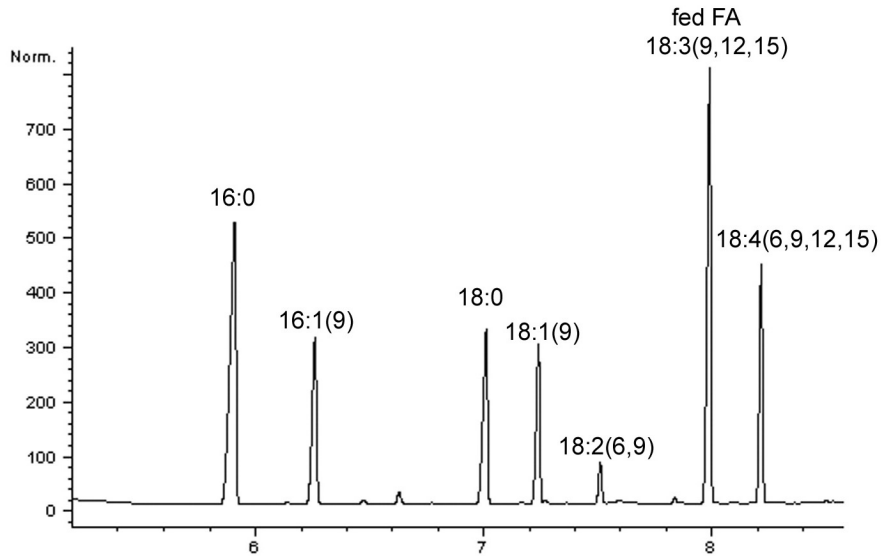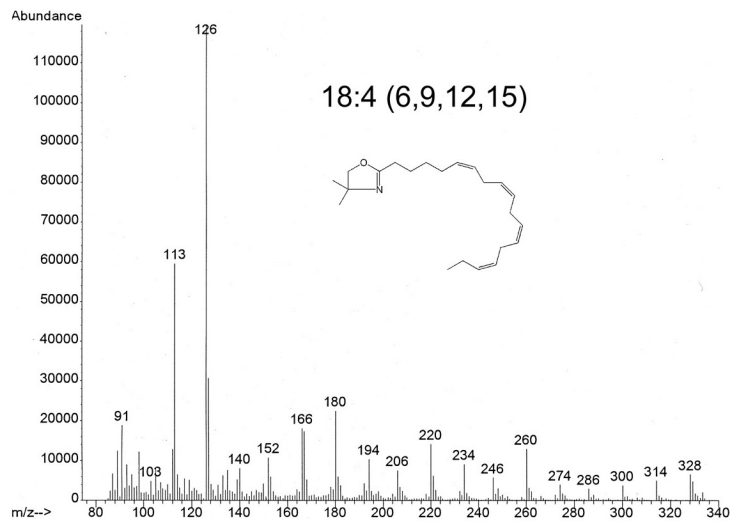
Figure 8. Identification of 18:4(6,9,12,15) in yeast. Yeast transformed with the pYES2 vector containing the delta6 desaturase of *Tetrahymena thermophila* and p414-GPD containing the AtCYB5-B cytochrome b5 of *Arabidopsis thaliana* were fed 18:3(9,12,15) and grown with galactose induction. (A) GC trace of total fatty acids including endogenous yeast fatty acids 16:0, 16:1, 18:0 and 18:1, fed fatty acid 18:3(9,12,15) and delta6 desaturation products 16:2, 18:2 and 18:4(6,9,12,15). (B) GC-MS identification of 18:4(6,9,12,15)
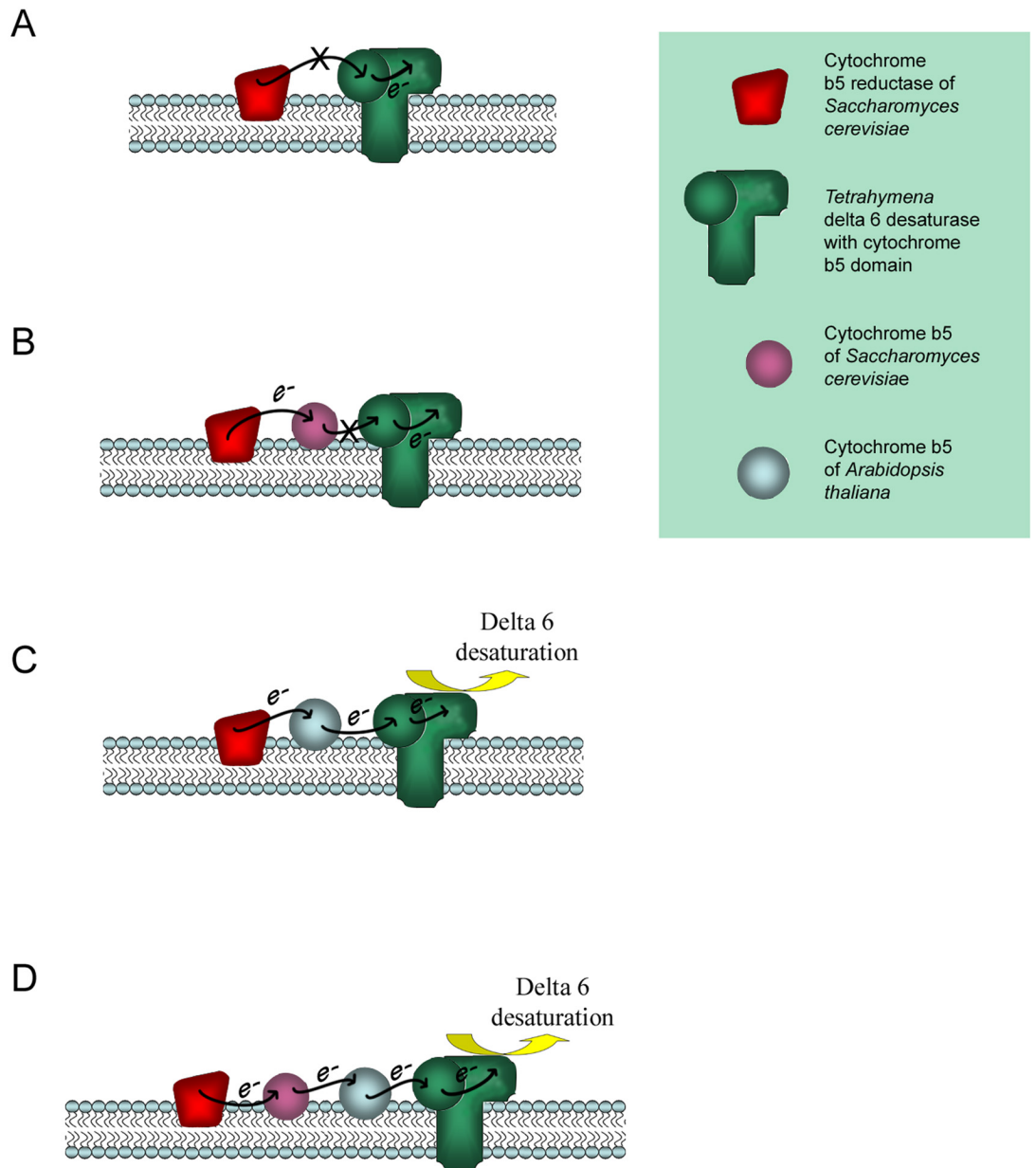
Figure 8. Potential pathways of electron transfer in the delta 6 desaturation pathway. In the absence of the *Arabidopsis* cytochrome b5 (A and B), there is limited delta 6 desaturation by the *T. thermophila* desaturase. Causes for the lack of desaturation could be the inability of the delta 6 cytochrome b5 domain to receive electrons from the yeast cytochrome b5 reductase (A) or from the yeast cytochrome b5(B). In the presence of the *Arabidopsis* cytochrome b5 (C and D), there is a 10 fold increase in delta 6 desaturation. This could be due to the *Arabidopsis* cytochrome receiving electrons from the yeast cytochrome b5 reductase and passing them on to the cytochrome b5 domain of the desaturase (C) or from the cytochrome b5 of yeast (D).

<center>**CHAPTER THREE**</center>

<center>THE DELTA 12 DESATURASE HOMOLOGS OF *TETRAHYMENA PYRIFORMIS*</center>

<center>AND *TETRAHYMENA THERMOPHILA*</center>

<u>**Introduction**</u>

The role of omega 3 fatty acids in human health and disease has been extensively investigated. Of the important omega 3 (ω3) fatty acids, the 20 carbon eicosapentaenoic acid (EPA, 20:5 (5,8,11,14,17)) and 22 carbon docosahexaenoic acid (DHA), 22:6 (4,7,10,13,16,19)) are those whose effects are most studied. Not only do these fatty acids play a role in membrane function and fluidity, but they have also been revealed to be beneficial in the prevention and treatment of certain human diseases, based on in vitro and epidemiological studies. Higher intake of these ω3 fatty acids decreases the incidence of human diseases such as heart disease, type II diabetes, and certain types of cancer. (Beck et al., 1991; Lo et al., 1999; Connor, 2000; Hughes-Fulford et al., 2001) .

The ω3 fatty acids are distinguished from other fatty acids by the presence of a terminal double bond three carbons from the methyl end of the molecule. Double bonds counted from the methyl end of the molecule are referred to as omega (ω) or n double bonds. Another group of polyunsaturated fatty acids are the omega 6 (ω6) fatty acids, which have their terminal double bond at 6 carbons from the end of the molecule. It is believed that the balance between these two families of fatty acids is important to human health, and that the fatty acids ingested in a normal Western diet are overbalanced in favor of ω6 fatty acids. (Simopoulos, 2003)

Most fatty acids in human diets are from cultivated crop plants and grain-fed meat. It is due to the high amount of ω6 fatty acids in plants that there is an

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overabundance of those fatty acids in our diet. These imbalances of ω6 relative to ω3 fatty acids cause problems since these fatty acids are precursors to important signaling molecules. The ω3 fatty acids, mainly EPA, are precursors for the 5-leukotrienes and 3-prostaglandins. Omega 6 fatty acids are precursors for the 4-leukotrienes and 2-prostaglandins. (Van et al., 1964; Hammarstrom, 1980) In the response to inflammation, ω3 fatty acids act to down regulate the expression of the cyclooxygenase (COX-2) necessary in the biosynthesis of prostaglandins and as competitors to the ω6 fatty acids for access to the COX-2. The ω6 fatty acid derived signaling molecules aid in inflammation and clotting. (Anggard and Samuelsson, 1965) The imbalance of ω6/ω3 fatty acid derived metabolites has been shown to play roles in cardiovascular disease, autoimmune diseases, and certain types of cancer. This imbalance is often treated with supplementation of ω3 fatty acids.

One current source of supplementation of ω3 fatty acids is from fish oil. However, there are drawbacks to harvesting these fatty acids from fish. Firstly, the presence of contaminants in fish is well documented. One of the more prominent contaminants is mercury, but there are also detrimental organic compounds such as dioxins and polychlorinated biphenyls. (Yokoo et al., 2003; Hites et al., 2004) Along with contamination problems, the population of fish worldwide is decreasing, so a more renewable resource would be preferred. One such source would be the production of EPA in oil seed crops. It has been shown that EPA can be made in transgenic *Arabidopsis* by the heterologous expression of three fatty acid modification enzymes. First a fatty acid elongase catalyzed the production of 20:3(11,14,17) from18:3(9,12,15). This elongation was followed by desaturation with the Δ8 desaturase from *Euglena*

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gracilis and the Δ5 desaturase from *Caenorhabditis elegans*. (Wallis and Browse, 1999; Watts and Browse, 1999; Qi et al., 2004; Domergue et al., 2005)   While this fatty acid modification scheme produces EPA in seeds, it does not make use of the large pool (around 15% total fatty acids) of 20:1(11) found in *Arabidopsis* seeds. If this fatty acid were to be used as a substrate for synthesis of EPA it would require the introduction of four fatty acid desaturases into *Arabidopsis thaliana* (Fig 8B).   These include the Δ5 and Δ8 desaturases previously mentioned, but also an ω3 desaturase from *C. elegans* (Spychalla et al., 1997), and a proposed Δ14 desaturase from *Tetrahymena pyriformis*.

Delta 14 desaturase activity has been described in *Tetrahymena pyriformis*, but the cDNA encoding the enzyme responsible for this unique activity has not been identified. In a paper by Lees and Korn and published in 1966, it was shown that Tetrahymena fed 20:1(11) as a substrate would produce 20:2(11,14). *Tetrahymena pyriformis* do not normally produce 20-carbon fatty acids. Most probably the Δ14 activity is due to the Δ12 desaturase, which normally acts on 18-C fatty acids, acting as a Δ14 desaturase on 20-C fatty acids. *Tetrahymena* do make a range of 16 and 18 carbon fatty acids, the synthesis of which would require the presence of Δ6, Δ9, and Δ12 desaturases. If the Δ12 desaturase of *Tetrahymena pyriformis* acts as an omega 6 desaturase, then the same enzyme could be responsible for the Δ12 and Δ14 desaturase activity.

The sequence of the Δ12 desaturase of *Tetrahymena* has not been published, and at the beginning of this project a comprehensive collection of *Tetrahymena* ESTs was not available. To identify the possible sequence of the *Tetrahymena* Δ12 desaturase, other members of the Δ12 family were examined. Delta 12 fatty acid desaturases from

disparate organisms exhibit sequence conservation, especially around the three histidine boxes characteristic of these proteins. By using regions of high sequence identity, degenerate primers can be designed using the sequence of known genes to amplify homologs of unknown sequence.

In this chapter I describe my efforts to clone and characterize the putative $\Delta12/\Delta14$ desaturase of *Tetrahymena*. Using the sequence of FAT-2, the $\Delta12$ desaturase of *C. elegans,* primers were designed to amplify the $\Delta12$ homolog of *Tetrahymena pyriformis*. Once a possible cDNA was identified, the cDNA was modified based on the difference in stop codon usage between *Tetrahymena* and the expression systems used. Following is the description of the cloning, mutation, modification, and expression of the *Tetrahymena* $\Delta12$ desaturase homolog in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* and the small amount of $\Delta12$ desaturase activity seen following codon optimization.

Materials And Methods

Verification of Δ14 activity in *Tetrahymena*

Five strains of *Tetrahymena* were acquired from the American Type Culture Collection including *Tetrahymena pyriformis* strains T and W (30202 and 10542), *Tetrahymena corlissi* (50086), and *Tetrahymena thermophila* strains A and I (30305 and 30847). To examine the Δ14 desaturation activity, exogenous fatty acids were fed to *Tetrahymena* cultures. The fatty acids used in this work were obtained as free fatty acids (NuCheck Prep, Elysian, MN). The fatty acids were weighed and dissolved in DMSO to a stock concentration of 100mg/ml. The *Tetrahymena* strains were grown as liquid cultures at 25º on a rotary shaker in an optimized media (Jim Wallis, personal comm.). Liquid cultures of *Tetrahymena* were fed 20:1 as a free fatty acid at a final concentration of 200μM. Log cultures that had been grown in media containing fatty acid were harvested after 2 days of growth by centrifugation followed by a series of three washes in sterile H_2O. Collected cells were then treated with H_2SO_4 in methanol to form fatty acid methyl esters as previously described. (Miquel and Browse, 1992) The hexane extractions of these FAMES were then analyzed on the Supelco SP-2380 narrow bore column by gas chromatography with a flame ionization detector (Agilent, Palp Alto, CA). To perform mass spectrometry analysis of the fatty acid, DMOX derivatization of the methyl ester was done as described (Fay and Richli, 1991). The identity of the product of Δ14 desaturation was then verified by gas chromatography with a mass spectrometry detector (GC-MS). Positive identification was done by comparisons with the spectra of known substrates.

Cloning of the Δ14 desaturase from *T. pyriformis*

Cloning of the Δ14 desaturase from *Tetrahymena pyriformis* RNA was done by James Wallis essentially as described for the Δ8 desaturase from *Euglena gracilis*. (Wallis and Browse, 1999) Degenerate primers used in the amplification of the Δ14 were designed to the *C. elegans* Δ12 desaturase, FAT-2. Primers were designed in regions of high conservation within the Δ12 desaturase family. RNA from *T. pyriformis* cells was isolated using TRIZOL reagent according to protocol used for the initial amplification, and for the subsequent rounds of rapid amplification of cDNA ends (RACE). The full length cDNA was then mutagenized to remove premature stop codons. This mutagenesis was performed as described for the Δ6 and Δ9 desaturases.

Cloning of the Δ14 desaturase from *T. thermophila*

A search of the Tetra Sequencing Project (http://cbr-rbc.nrc-cnrc.gc.ca/reith/tetra/ tetra.html) led to a *Tetrahymena thermophila* EST, 5009-0-41-A07, with homology to known Δ12 desaturases. The EST sequence was used to design the forward cDNA-specific primers Th12EST-254F, Th12EST-438F and Th12EST-634F and the reverse primers Th12EST-96R, Th12EST-342R, and Th12EST-547R (Table 12-1). The cDNA library used as a template for amplification of the target cDNA was received from Aaron Turkewitz. For amplification, the cDNA-specific primers were paired with either a T7 or a M13R primer which are vector specific. Amplification used KOD polymerase and the same reaction and thermocycler program as for the amplification of the Δ6 and Δ9 cDNAs. These PCR products were analyzed to obtain the sequence of the 5' and 3' ends. Primers designed from the far 5' and 3' cDNA sequence were used to amplify out the full length PCR product. This cDNA was then cloned into a vector for further analysis and

the resultant sequence was compared to other Δ^{12} desaturases. The cDNA was also mutagenized to remove excess stop codons using the protocol used for the other desaturases.

Southern blot of *Tetrahymena* genomic DNA

Genomic DNA was isolated from the five Tetrahymena strains to perform a Southern Blot. DNA was isolated using a phenol-chloroform extraction, and harvested by spooling on a glass rod. DNA was then restricted with EcoRI, EcoRV, HindIII, SalI or XbaI and electrophoresed on a 0.8% agarose gel, with a partial length and the full length tdm-1 cDNA as a positive control. Following visualization of the gel, the DNA was transferred to a nitrocellulose membrane by wicking. After transfer, the DNA was permanently linked to the membrane using a UV cross-linker. The probe for the Southern blot was generated by PCR amplification of the full length tdm-1 cDNA using P^{32} dCTP according to published protocols. The blot was pre-hybridized in ULTRAhyb buffer prior to the addition of the radiolabeled probe and also acted as the hybridization buffer following the published protocols. (Ambion, Inc. Austin, TX). The blot was then washed with a low stringency protocol and then placed on Whatman paper and wrapped in plastic wrap. The blot was exposed on Kodak x-ray film at -80°.

Transformation of tdm-1 into *Saccharomyces cerevisiae*

The cDNA that was isolated from *Tetrahymena pyriformis* and mutagenized was labeled tdm-1 (*T*etrahymena <u>d</u>esaturase <u>m</u>utant-1). In an attempt to verify that this was the correct cDNA to account for the $\Delta14$ desaturation seen in *Tetrahymena*, it was heterologously expressed in *Saccharomyces cerevisiae*. The cDNA was first sub-cloned

from the sequencing vector pCR-script into the yeast expression vector pYES2. This

vector has a URA selectable marker and a galactose inducible promoter – GAL1. This

vector was constructed by the double restriction of tdm-1 and pYES2 with BamHI and

XhoI (New England Biolabs) followed by the ligation of tdm-1 into pYES2. This vector

was then sequentially transformed into *E. coli* and then into *S. cerevisiae*. The yeast cells

used were the Invitrogen S. c. cells that were made competent by the Invitrogen yeast

transformation kit. After transformation, yeast cells containing the vector were selected

for on media lacking uracil. Transformed yeasts were used to start liquid cultures that

were grown in the presence of fatty acids that were substrates for the expected

desaturation activity. Fatty acids were added to SD-ura media to a final concentration of

200μM and were added in the form of free fatty acids in solution in DMSO. Additional

DMSO was added to the media to a final 1% (v/v) concentration in order to aid uptake of

the fatty acid, this DMSO was added whether the yeast were fed fatty acid or not. Fatty

acids used were 18:1(9) and 20:1(11). Yeast cultures were grown for a day and then the

yeast cells were collected by centrifugation. Cells for each treatment, fatty acid fed or

unfed, were then re-suspended in a small amount of SD Gal –ura media and divided into

two sterile flasks. The two aliquots were then re-suspended in either galactose media or

glucose media, in order to look at the effect of galactose induction. These cultures were

then grown for an additional day or two and then examined by GC. Cells were collected

for GC by a series of centrifugation and wash steps. After the final centrifugation and

pouring off of the supernatant the cells were treated with H_2SO_4 in methanol as

previously described. The hexane extractions of the FAMEs were run on the gas

chromatograph with a flame ionization detector to find the fatty acids present in the yeast. These peaks were compared to standards of known fatty acids.

Northern blot of yeast expressing tdm-1

RNA was isolated from yeast cultures described above including galactose induced yeast with the cDNA behind the GAL1 promoter, and yeast with the cDNA behind the TEF and GPD promoters, which are constitutive. Yeast were grown to log phase, harvested by centrifugation, and Trizol was used to isolate their RNA according to the published protocol. The concentration of RNA was analyzed by a UV-spectrophotometer and equal concentrations of RNA were loaded on a formaldehyde agarose gel as previously described. Following electrophoresis the gel was blotted onto a nitrocellulose membrane using the TurboBlotter system. (Schleicher and Schuell) Once the RNA was transferred fully to the membrane it was attached to the membrane with a UV cross-linker. The membrane was then pre-hybridized in ULTRAhyb buffer before addition of the probe. The probe for the Northern was the same as was generated for the Southern Blot. The membrane was hybridized with the probe and was then washed with high stringency. The radiolabeled probe hybridized to the membrane was then visualized on film.

Genomic sequence of *Tetrahymena* delta 14 desaturase

Using the far 5' and 3' primers used to amplify the full-length cDNA, the genomic region between the start codon and the terminal TGA was amplified. This product was then sequenced using primers that were internal to the tdm-1 cDNA. The genomic sequence and the cDNA sequence were then aligned and analyzed for introns and exons and sequence identity.

Modification of the *Tetrahymena* desaturase

Addition of an ER retention signal to tdm-1 was done using a modified 3' primer. The cDNA was amplified as before but with the addition of sequence to the 3'end. Alteration of the first codons in the cDNAs was done using a modified 5' primer. Codon changes were designed into the primer which was then used for cDNA amplification.

Transformation of tdm-1 into *Caenorhabditis elegans*

A fusion cDNA of tdm-1 and green fluorescent protein was constructed for transformation into C. elegans. This fusion was expressed using the promoter for the *C. elegans* omega-3 desaturase fat-1. The three pieces used, the promoter, tdm-1 and gfp, were amplified into one fragment using the protocol previously described for removal of unwanted stop codons. The full length construct was then used in microinjection of the *lin*-15 mutant *C. elegans* as previously described (ref). The plasmid pJM23 containing the lin-15 gene was co-injected with the construct to act as a marker for successful transformation. Rescued worms were used for further study. Non-rescued *lin*-15 worms grown at 25º develop a multi-vulva phenotype, and rescued worms appear wild type. After injection, surviving worms were separated onto individual plates and put at 25º and grown to the next generation and further checked for heritability of the *lin*-15 rescue. Worms that appeared WT for two generations were then examined for GFP expression. If GFP expression was seen in a transformed line, the worms were fed 20:1, the fatty acid substrate for Δ14 desaturation. Worms were plated on media containing the fatty acid

and seeded with E. coli as a food source. Fatty acids of the transformed worms were examined by gas chromatography as previously described.

Transformation of tdm-1 into *Arabidopsis thaliana*

To examine the expression of tdm-1 was in *Arabidopsis thaliana*, tdm-1 was sub-cloned from pCR-script into pOEA2 and into pSSP7. The pOEA2 vector is complete for the necessities of plant expression including the phaseolin promoter, the NOS terminator, the Basta selectable marker, and the left and right borders. The napin containing pSSP7 vector was used in conjunction with the pART27 and pBART vectors. After cloning tdm-1 into pSSP7, the promoter::cDNA::terminator fragment was cloned into These vectors contain the left and right borders and the plant selectable markers, kanamycin resistance in pART27 and Basta resistance in pBART. The complete constructs were then used to transform Agrobacterium, and transformed cells were selected on plates containing spectinomycin (the bacterial selection used in all three vectors), and gentamicin (the selectable marker for the Ti plasmid. *Agrobacteria* containing the various constructs were then grown and used to transform wild type *Arabidopsis* via the Floral Dip method. These original transformed plants are referred to as the T_0 population. Seeds from these transformed plants, T_1 seed, was then plated on MS plates containing kanamycin for the pART27 transformed plants, or sown on Basta containing soil for the pBART or pOEA2 transformed plants.   The resistant T_1 plants were then grown and seed was collected. This T_2 seed was then FAME analyzed by GC for any changes in fatty acid profile.

Results and Discussion

Δ14 activity in *Tetrahymena*

Tetrahymena produce endogenous fatty acids that should require Δ6, Δ9 and Δ12 desaturases for their synthesis. The fatty acids of *Tetrahymena* include the saturates 16:0 and 18:0 as well as the desaturation products 16:1, 18:1, 18:2 and 18:3. Most *Tetrahymena* species, including *pyriformis* and *thermophila*, do not produce fatty acid chains longer than 18 carbons in detectable amounts. In order to verify the presence of a desaturase that would use a 20-carbon substrate, cultures of *Tetrahymena pyriformis* and *Tetrahymena thermophila* were grown in media containing 20:1(11) free fatty acid. *Tetrahymena* cells were harvested and the fatty acids were derivatized to FAMEs for analysis by gas chromatography. Unfed *Tetrahymena* had peaks corresponding to 16- and 18-carbon fatty acids that were products of Δ6, Δ9 and Δ12 desaturases, and did not contain any 20-carbon fatty acids. (Figure 11A) *Tetrahymena* cultures that were fed 20:1(11) had the normal 16- and 18-carbon fatty acids and also had a peak corresponding to the fed fatty acid and an additional peak not present in the unfed organisms. The additional peak had a GC retention time corresponding to a 20-carbon fatty acid with two double bonds. (Figure 11B) The FAMEs of the total *Tetrahymena* fatty acids were further derivatized to produce the DMOX derivatives. These derivatives were analyzed by GC-MS in order to verify the double bond position of the novel fatty acid produced. The identity of the novel 20:2 fatty acid was confirmed as 20:2(11,14) (Figure 11C), verifying the ability of *Tetrahymena* to desaturate 20:1(11) with the introduction of a Δ14 double bond. The 20:2(11,14) fatty acid appears to be a true desaturation product

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instead of the product of an elongation reaction since 20:2 is not seen in the lipids of *Tetrahymena* cells without feeding of 20:1(11).

Cloning of the full length cDNA

Once a Δ14 activity in *Tetrahymena* species was verified, the amplification of the cDNA coding for this desaturase was undertaken. As previously stated, most *Tetrahymena* species do not produce fatty acids chains longer than 18 carbons, and therefore would not be expected to have a desaturase that acts exclusively on 20-carbon fatty acids. However, these species do regularly desaturate 18-carbon fatty acids with a Δ12 desaturase. It is likely that this desaturase is also responsible for the Δ14 desaturation of 20:1. In both the Δ12 desaturation of 18:1(9), and the Δ14 desaturation of 20:1(11), a double bond is introduced 6 carbons from the methyl end of the molecule, in the ω6 position. Also, the second double bond would be inserted in a position three carbons from the existing double bond. With the reasoning that the Δ12 desaturase homolog from *Tetrahymena* could act as a Δ14 desaturase, work was begun to amplify the Δ12 desaturase from *Tetrahymena*.

The cDNA from *T. pyriformis* was amplified using degenerate primers designed based on the sequence of the Δ12 desaturase from *Caenorhabditis elegans*. (Peyou-Ndi et al., 2000) These primers were designed at regions of high conservation at the 1[st] and 3[rd] histidine boxes characteristic of this group of proteins. RNA isolated from *Tetrahymena pyriformis* was used as a template for reverse transcription followed by PCR amplification with degenerate primers. Further reactions using 5' and 3' rapid amplification of cDNA ends (RACE) allowed for the amplification of the full length cDNA. The cDNA that was isolated from *Tetrahymena pyriformis* was labeled td-1

(_Tetrahymena_ _d_esaturase-1).   The coding region of the cDNA predicted a protein

sequence of 375aa with a MW of 42.9 kD.

The cDNA of the Δ12 desaturase homolog was also cloned from _Tetrahymena_

thermophila. A partial database of _T. thermophila_ ESTs is available on the TIGR

website. When a BLAST search of the database was performed using the predicted

protein sequence of the _T. pyriformis_ Δ12 desaturase, an EST appearing to be a partial

Δ12 cDNA was recovered. Primers Th12EST-254F, 438F, 634F, 96R, 342R and 547R

(Table 3) were designed to the EST sequence and were used with vector primers M13

and M13R to amplify the cDNA from a _Tetrahymena thermophila_ cDNA library kindly

provided by Dr. Aaron Turkewitz. The _T. thermophila_ Δ12 desaturase cDNA (Tt del12)

was cloned into the pCR-script vector fully sequenced. Using a translation table for

ciliated protists, the Tt del12 cDNA is predicted to encode a protein of 371aa with a MW

of 42.6 kD.

Homology to known delta 12 desaturases

The predicted protein sequences of the _T. pyriformis_ and _T. thermophila_ cDNAs

were used in a BLAST search of the non-redundant protein database.   Both of the

Tetrahymena desaturases were found to be most closely related to the same members of

the Δ12 desaturase family. The two _Tetrahymena_ protein sequences returned the same

BLAST results due to the high identity between the two proteins which are 86% identical

and 93% similar. An alignment of the _Tetrahymena_ putative desaturases with the

Arabidopsis thaliana Δ12 (FAD2) (Okuley et al., 1994) and the _Caenorhabditis elegans_

Δ12 (FAT-2) (Peyou-Ndi et al., 2000) reveals similar protein length and relative positions

of the start methionines (Figure 12). The homology between the proteins in this family is

especially conserved in the regions of the three histidine boxes characteristic of membrane bound desaturases. These conserved domains are involved in the organization of iron molecules at the active site. The first of the three histidine boxes HECGH (conserved sequence HXXXH) begins at amino acid 99 for tdm-1 and 95 for th12. The second HHVHH (HXXHH) at residues 135 for tdm-1 and 131 for th12, and the last conserved histidine box is HVVHH (HXXXH) as found beginning with amino acids 315 and 311, respectively (underlined in Figure 12). These histidine boxes align with those of the *Arabidopsis* and *C. elegans* desaturases, and follow the conserved sequence necessary for the correct functioning of this family of desaturases.

S. cerevisiae **expression of the cDNA**

As explained in the previous chapter, *Tetrahymena* cDNAs often contain unwanted stop codons that needed to be removed by site directed mutagenesis. Each of the Δ12 cDNAs contained six unwanted stop codons. To eliminate these stop codons the cDNA sequence was changed using overlap extension PCR as described in Chapter Two. The cDNAs were then cloned into a sequencing vector and verified for the presence of the desired changes, and absence of unwanted changes.

To examine the activities of the Δ12-like proteins of *T. pyriformis* and *T. thermophila*, their cDNAs were sub-cloned from the pCR-script sequencing vector into the yeast expression vector pYES2. These constructs were then used to transform *Saccharomyces cerevisiae*. In order to provide a substrate for Δ14 desaturation that is not endogenously present in yeast, cultures were fed 20:1(11) as a free fatty acid. Yeast transformed with the empty vector of pYES2 was used as a control. Cultures were grown in the presence or absence of the fatty acid and some were induced with galactose. Fatty

acids were then analyzed by methyl ester derivatization followed by gas chromatography. Yeast cultures were triple washed before analysis to ensure that fatty acids examined had been incorporated into the yeast, and were not just present in the media. Yeast that was fed 20:1 assimilated significant amounts of the fatty acid into its lipids. However, even with an available pool of Δ14 desaturase substrate, yeast transformed with the desaturase cDNAs and induced by galactose did not have a fatty acid profile containing any polyunsaturated 20-C fatty acids. Desaturase activity also was not seen by the Δ12 desaturase acting on endogenous 18:1 (9).

The identity of this cDNA as coding for a Δ12 desaturase was assigned due to its similarity to other Δ12 desaturases, but it was possible that a different Δ12 homolog encoded the desired desaturase.

Search for homologs of td-1 in *Tetrahymena* species

Working with the theory that the Δ14 activity is a result of a promiscuous Δ12 desaturase, we would not expect another desaturase in *Tetrahymena pyriformis* to have homology to the cloned Δ12. Examination of the available *Tetrahymena* ESTs did not reveal any desaturases other than the expected Δ6, Δ9, and Δ12 desaturases, but if *Tetrahymena* contained another desaturase gene than those seen in EST analysis, it could be recognized in a Southern blot of *Tetrahymena* genomic DNA. This is based on the assumption that a gene encoding the Δ14 desaturase would be homologous to the cDNA for the Δ12 desaturase that was previously isolated. Southern blots of digested DNA from five different strains of *Tetrahymena, T. pyriformis* T and W, *T. corlissi* and *T. thermophila* A and I, were probed with the full length radiolabeled tdm-1 cDNA (Figure 11). Multiple bands were predicted for EcoRI, EcoRV, and HindIII based on cut sites in

the cDNA. Single bands are seen for the SalI and XbaI lanes. Low stringency washing protocols were used in order to try to identify genes homologous to tdm-1 that could account for the Δ14 desaturase activity of *Tetrahymena pyriformis*. The genomic regions containing the td-1 gene were identified in all five *Tetrahymena* species examined, with the strongest binding in *pyriformis* T and W. Banding patterns were those as expected for a single Δ12 desaturase like gene in the *Tetrahymena* species examined. Blots were overexposed to identify any faint bands that could correspond to another homolog of the Δ12 desaturase, but none were found.

Because the cDNAs that we have apparently code for the only Δ12 desaturase of *Tetrahymena*, other potential problems in the heterologous expression in yeast were considered. The presence of a desaturase alone may not be enough to ensure desaturation, as was seen in the previous chapter. The enzyme needs to be correctly inserted into the membrane, and it needs iron, oxygen, and an electron donor to function correctly. And there is also the possibility that the protein is not present at all since the cDNA needs to be properly transcribed and translated.

Verification of the presence of tdm-1 message in *S. cerevisiae*

One possible explanation for the lack of desaturase activity in *S. cerevisiae* is if the message for the desaturase protein is not transcribed. To address this issue radio-labeled tdm-1 cDNA was used to perform a Northern Blot on RNA isolated from transformed and non-transformed yeast. RNA was isolated from 4 different yeast strains. Yeast containing the pYES2 vector with or without the tdm-1 transgene were treated with galactose to induce transcription with the GAL1 promoter. RNA was then isolated by Trizol extraction from these strains. RNA was also collected from yeast expressing the

tdm-1 transgene under the control of either of two constitutive promoters, TEF or GPD. The transcript detected was ~1.2 kb, corresponding to the predicted size of the transcript, and was detected in yeast regardless of which of the three promoters were driving transcription (Figure 14). However, the transcript was most abundant in yeast where the cDNA transcription was driven by the GAL1 promoter. With the abundance of message found by Northern Blot, it does not appear that the lack of desaturation activity is caused by lack of cDNA transcription.

We expect with transcription of the cDNA into mRNA, translation will also occur, but the presence of protein has not been verified in yeast. However there are other possible problems with the desaturase being heterologously expressed. With the mRNA present but no activity of the desaturase in yeast, verification of the length and sequence of the cDNA was undertaken.

Search for upstream coding sequence for td-1

Desaturases need electrons in order to catalyze the formation of the double bond. For many desaturases, including all known members of the FAD2 super-family, the protein that donates that electron is a cytochrome b_5. Some desaturases contain a cytochrome b_5 domain as a part of the protein, like members of the $\Delta5/\Delta6$ desaturase family. If there is a cytochrome b_5 domain that should be a part of the *Tetrahymena* desaturase that is not present in tdm-1, it could account for why no activity is seen. Initial work was done using RNA amplification techniques to identify a longer cDNA, but a longer sequence was not found. These included reverse transcription in the presence of trehalose to increase the length of the RT product, and an RNA ligase mediated RACE to ensure amplification of the full length mRNA. Further work was then

undertaken using *Tetrahymena* genomic DNA to identify any putative upstream coding sequence. In order to verify that the 5' end of the tdm-1 cDNA is full length, TAIL-PCR (thermo asymmetric interlaced polymerase chain reaction) was performed to amplify the genomic DNA flanking the 5' of the tdm-1 sequence. A total of approximately 2kb upstream of the ATG start codon was amplified and sequenced. This sequence was analyzed for the presence of a cytochrome b_5 domain, or for the presence of apparent coding sequence representing additional codons belonging to the desaturase. The sequence that was identified did not have a cytochrome b_5 domain, and did not appear to contain significant coding sequence. The sequence was also used as a query in a BLAST search of the non-redundant database, and the region was not identified as homologous to any known protein. These results indicate that this region does not contain additional coding sequence for the desaturase.

Comparison with genomic sequence to ensure the cDNA is correct

Another possible issue with the protein could be mutations introduced into the cDNA sequence during RT-PCR. To test this possibility, the genomic DNA encoding the Δ12 desaturase homolog was amplified out of *Tetrahymena pyriformis* and sequenced. This alignment revealed the presence of three introns in the coding region of td-1, all of which are flanked by highly likely donor and acceptor sites when the sequence is analyzed by an intron prediction program. The sequences of the predicted introns were analyzed for possible coding sequence. The predicted introns did not contain any sequence that was identifiable as coding sequence for known desaturases or other known proteins. When the sequences were aligned without the predicted introns there is no difference in the sequence between the genomic DNA and the coding sequence of the

tdm-1, other than the changes introduced by site-directed mutagenesis to remove unwanted stop codons from the cDNA. With the analysis of the length of tdm-1 and the sequence confirmation, I believe that this is the correct sequence for this desaturase.

Through these varied studies we found that the Δ12 homolog we were using was the correct length for a Δ12 desaturase, and when aligned with other members of the same family, conserved regions were identified and no gaps were found in the sequence. By analysis of the genomic DNA encoding the cDNA and the DNA flanking the 5' region it appears that the cDNA is the correct sequence and of the correct length. However, transgenic yeast containing the Δ12 desaturase homolog did not show any Δ12 or Δ14 activity.

Modifying the retention of tdm-1 cDNA to attempt to see heterologous expression

Many membrane bound desaturases are localized in the endoplasmic reticulum, and some desaturases have recognizable ER retention signals as a part of their sequence. This can be seen in the *C. elegans* protein FAT-2 which has the C-terminal sequence KKAQ which follows with the canonical ER retention sequence KKXX. To help ensure the proper localization of tdm-1 (the Δ12 cDNA of *T. pyriformis*) to the ER when it is expressed in yeast, the cDNA sequence was altered to include an ER retention sequence in its protein product. This ER retention signal was added in two different ways. In one experiment codons for an additional three amino acids were added onto the end of tdm-1 changing the sequence coded for at the end of the protein from ….YYWK to ….YYWKKAQ. In a second experiment the coding region for the last 4 amino acids of tdm-1 was exchanged for the sequence coding for the last 16 amino acids of FAT-2. This

changed the TDM-1 protein sequence from ….YYWK to ….LQYRSGVEAAKAKKAQ. These two modified cDNAs were then cloned into pCR-script for sequencing and then sub cloned into the yeast expression vector pYES2. The pYES2 constructs were used to transform competent yeast to look for the presence of either Δ12 or Δ14 desaturation. Transformed yeast were fed 20:1 and induced with galactose to look for desaturase activity as seen by the presence of novel fatty acids. Yeast containing these modified cDNAs did not produce any novel fatty acids, and therefore did not appear to have any desaturase activity other than that accounted for by the endogenous Δ9 desaturase.

Codon optimized cDNAs

When *Tetrahymena* Δ12 cDNAs were analyzed for their codon usage, there were no obvious codon problems for expressing the proteins in the species *S. cerevisiae*, *C. elegans*, or *A. thaliana*. This was determined by identifying rare codons of the expression systems and verifying their absence in the *Tetrahymena* cDNAs. While rare codons were not found, the codons could still be optimized to see if there was an affect on desaturase activity in yeast. In order to address this issue, the first 10 codons of tdm-1 and the first 12 codons of Ttdel12 cDNAs were changed to the most common codons used by *Arabidopsis thaliana* for each amino acid residue. With these changes, the codon usage was also nearly optimized for expression in *S. cerevisiae*, which has codon usage close to that of *Arabidopsis*. Only codons early in the sequence were modified, based on the assumption that once the ribosome is attached to the mRNA and has started translation, that the entire protein will be made. These modified cDNAs were cloned into pCR-script for sequencing and then sub cloned into pYES2 for yeast expression. Cultures of yeast transformed with the codon optimized cDNAs in pYES2 were fed 20:1

and induced with galactose in order to examine the lack or presence of Δ12 or Δ14 desaturation products. Fatty acids were analyzed by gas chromatography. Samples that were galactose induced did contain a peak corresponding to a new fatty acid running at a retention time indicative of it being 18:2(9,12), this peak represented only about 0.5% of the total yeast fatty acids, but was highly reproducible. (Figure 15) The presence of the peak confirms that the cDNAs used do code for a protein that has Δ12 activity, even if the activity is low in yeast. Samples that were fed 20:1 and induced did not have a peak corresponding to the presence of the Δ14 desaturation product, 20:2.

In *Tetrahymena* species, the Δ12 desaturase is highly active, but again enzymes cannot always be counted on to have the same activity in heterologous systems as they have in the host organism. I attempted to increase the small amount of Δ12 desaturation activity seen when the codon optimized cDNAs were expressed in yeast by co-expression with an Arabidopsis cytochrome b5. Co-expression did not increase the amount of desaturation seen in yeast. To continue to examine the role of the *Tetrahymena* Δ12 homolog, two other expression systems were used.

C. elegans expression of the cDNA

Expression of the Δ12-like cDNAs in *S. cerevisiae* did not verify that these cDNAs encode the protein responsible for Δ14 desaturation activity seen in *Tetrahymena*. To examine whether the lack of activity is due to incompatibility with the yeast expression system, tdm-1 was also transformed into *C. elegans*. In order to transform nematodes, overlap extension PCR was used to make a fusion fragment including the fat-1 promoter of *C. elegans* followed by the tdm-1 cDNA (without the stop codon) and the DNA sequence encoding GFP at the 3' end. This fusion was assembled using PCR and

overlapping primers such as in the site-directed mutagenesis. This construct was then microinjected into young adult hermaphroditic *C. elegans* along with a rescue plasmid to identify transformed individuals. After transformation worms were separated onto individual plates for examination of future generations. Offspring stable for expression of the rescue plasmid were then examined for GFP expression. GFP expression was seen in the worm lines examined. The GFP expression was localized in the intestine, the neurons, or both, which is expected for a transgene driven by the fat-1 promoter (Figure 16A-C). GFP fluorescence was not seen in non-transformed worms (Figure 16D) The worms that had GFP expression were then moved to plates containing various concentrations of the fatty acid 20:1(11). Fed (20:1) and unfed worms were harvested and analyzed by methyl ester derivatization and gas chromatography. The amount of 20:2 present in nematodes fed 20:1(11) or unfed was analyzed for both WT worms and worms transformed with the tdm-1 construct. Fatty acid traces of worms containing tdm-1 and fed 20:1(11) did not have a higher amount of 20:2(11, 14) than WT *C. elegans*, indicating a lack of $\Delta 14$ desaturation.

The other possibility besides the inactivity of tdm-1 as a $\Delta 14$ desaturase is that the 20:1 is desaturated to 20:2(11,14) and it is used immediately as a substrate for further desaturation. The indication that this is not the case is found in the lack of an increase in the percentages of polyunsaturated 20-carbon fatty acids.

Expression of the *Tetrahymena* Δ12 homolog in *Arabidopsis*

The final heterologous system used for expression of the tdm-1 desaturase was *Arabidopsis thaliana*. For plant transformation tdm-1 was sub-cloned from pCR-script into pART27 and pBART via pSSP7 and into pOEA2. The pART27 and pBART vectors

both use the napin promoter, which highly expresses a seed-storage protein in its native *Brassica napus*, and also acts to highly express transgenes in seeds of other species including *Arabidopsis*. The pOEA2 vector contains the phaseolin promoter from *Phaseolus vulgaris*, which like napin is a seed-specific promoter that will highly express transgenes in seeds. These vectors were used to transform Columbia WT *Arabidopsis* and the mutant *fad2-2* using the floral dip method. Transformed seeds were screened for by planting on either Basta treated soil (plants transformed with either the pBART or pOEA2 vectors), or by plating out seeds on MS plates containing kanamycin (plants transformed with pART27). The seeds from primary transformants were examined by methyl ester derivatization and gas chromatography. *Arabidopsis* does have a small amount of endogenous 20:2, due to elongation of 18:2(9,12), so activity of a delta 14 desaturase would be identified by an increase in the amount of 20:2 (11,14) in the lipid profile. An increase in the amount of 20:2 was not found in the seeds of the primary transformants of *Arabidopsi*s containing the tdm-1 transgene or in further generations analyzed.

Conclusion

The desaturase encoded by tdm-1 was analyzed and modified and heterologously expressed in three different systems, yeast, nematode, and plant. A small amount of activity that was seen in yeast expressing codon-modified *Tetrahymena* cDNAs, but the small amount of activity seen was that of a $\Delta 12$ desaturase and not of a $\Delta 14$ desaturase. The other expression systems, *C. elegans* and *A. thaliana*, have endogenous $\Delta 12$ desaturases so if the *Tetrahymena* desaturase was active on 18:1, the desaturation product could not be distinguished from the naturally occurring 18:2(9,12). However, the unique activity this $\Delta 12$ homolog was expected to have was as a $\Delta 14$ desaturase for 20-C fatty acids. This activity was not seen in the yeast, nematode or plant expression systems.

Table 3. Primers used in the amplification and mutation of the Δ12 desaturase homologs from *Tetrahymena pyriformis* and *Tetrahymena thermophila.*

	Primer name		Primer sequence 5' – 3'
Delta 12 T. th	Th12EST-254F		GCTGATAGATATCTCCGTTTAGAATGGGCC
	Th12EST-438F		CCTTACTTCTCTTGGTAAAGGTCTCATCATG
	Th12EST-634F		GGCTGCCTATCTATATAGGGGCTAAGGTGG
	Th12EST-96R		CCGTTAAAAACTGTATCGTTTTCAATGGAGC
	Th12EST-342R		GCTTTGTGACCACATTCGTGACCTAAAACC
	Th12EST-547R		CCAATAATATCTTCTAAGCTTGATATACATAG

	Primer name	Mutagenesis site	Primer sequence 5'--3'
Delta 12 T. py	tdm-1 coop 5'RE		GGTACCATGTCTAATACTTCTGCTAAGAAGTCTGATACTCAAACTGCTAT
	tetFM290	T259C	GCTTATGCAGTTATTCAAGGTACTATTGCC
	tetRM290	T259C	GGCAATAGTACCTTGAATAACTGCATAAGC
	tetFM410	T394C	TTTGGTTCCTTACTTCTCCTGGCAATGGTCTCACCACGTTC
	tetRM410	T394C	GAACGTGGTGAGACCATTGCCAGGAGAAGTAAGGAACCAAA
	tetFM580	T538C	CTTTCTCCATTTGGCAGATCCTCAACATCCTC
	tetRM580	T538C	GAGGATGTTGAGGATCTGCCAAATGGAGAAAG
	tetFM850	T814C	GGTATTACTTTCCTCCAACACACTGAAGACACCGTTCCTC
	tetRM850	T814C	GAGGAACGGTGTCTTCAGTGTGTTGGAGGAAAGTAATACC
	tetFM925	T891C	TTTGTGCACCATTGACAGAAACTACCCTGAATTC
	tetRM925	T891C	GAATTCAGGGTAGTTTCTGTCAATGGTGCACAAA
	tetFM1040	T1012C	AACATCTACCTTAAGCAGATTCTCGGACCTGCTTAC
	tetRM1040	T1012C	GTAAGCAGGTCCGAGAATCTGCTTAAGGTAGATGTT
	tdm-1 3'RE		
Delta 12 T. th	th12 5'RE	T13C	ATGACAAGCGCACAGACTAAAAAAAGCTCC
	th12 500F	T382C	CCTTACTTCTCTTGGCAATGGTCTCATCATG
	th12 500R	T382C	CATGATGAGACCATTGCCAAGAGAAGTAAGG
	th12 600F	T526C	CTTTCTCCATCTGGCAAGTTCTTAACATCTTAG
	th12 600R	T526C	CTAAGATGTTAAGAACTTGCCAGATGGAGAAAG
	th12 800F	T718C	CTATCTTTGGGCTAGACAAACTTCTTTCACC
	th12 800R	T718C	GGTGAAAGAAGTTTGTCTAGCCCAAAGATAG
	th12 1100F	T1000C	GCTAATATCTACCTCAAATAAATTCTCGGTCC
	th12 1100R	T1000C	GGACCGAGAATTTATTTGAGGTAGATATTAGC
	th12 3'RE	T1093C	TCATTTCCAATAATAGAAACCTTGTTCTCTTTCAGAG

A
known 20:5 desaturation
pathway

18:1
↓ Δ12
18:2(9,12)
↓ ω3
18:3(9,12,15)
↓ Δ6
18:4(6,9,12,15)
↓ elo
20:4(8,11,14,17)
↓ Δ5
20:5(5,8,11,14,17)

B
alternate
pathway

18:1
↓ elo
20:1(11)
↓ Δ14
20:2(11,14)
↓ Δ8
20:3(8,11,14)
↓ ω3
20:4(8,11,14,17)
↓ Δ5
20:5(5,8,11,14,17)
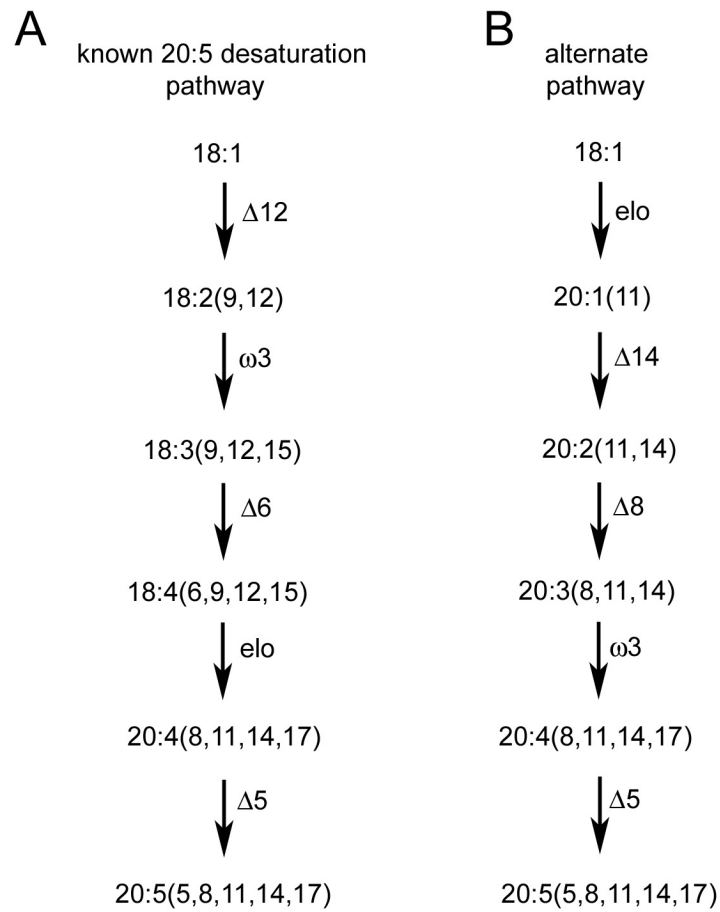
Figure 10. Pathways for the production of eicosapentaenoic acid. Known pathway for EPA synthesis (A) involving elongation of 18:4(6,9,12,15) to 20:4(8,11,14,17) followed by delta 5 desaturation. Proposed pathway for EPA biosynthesis involving a 20:1 intermediate and the delta 14 and delta 8 deaturases (B).
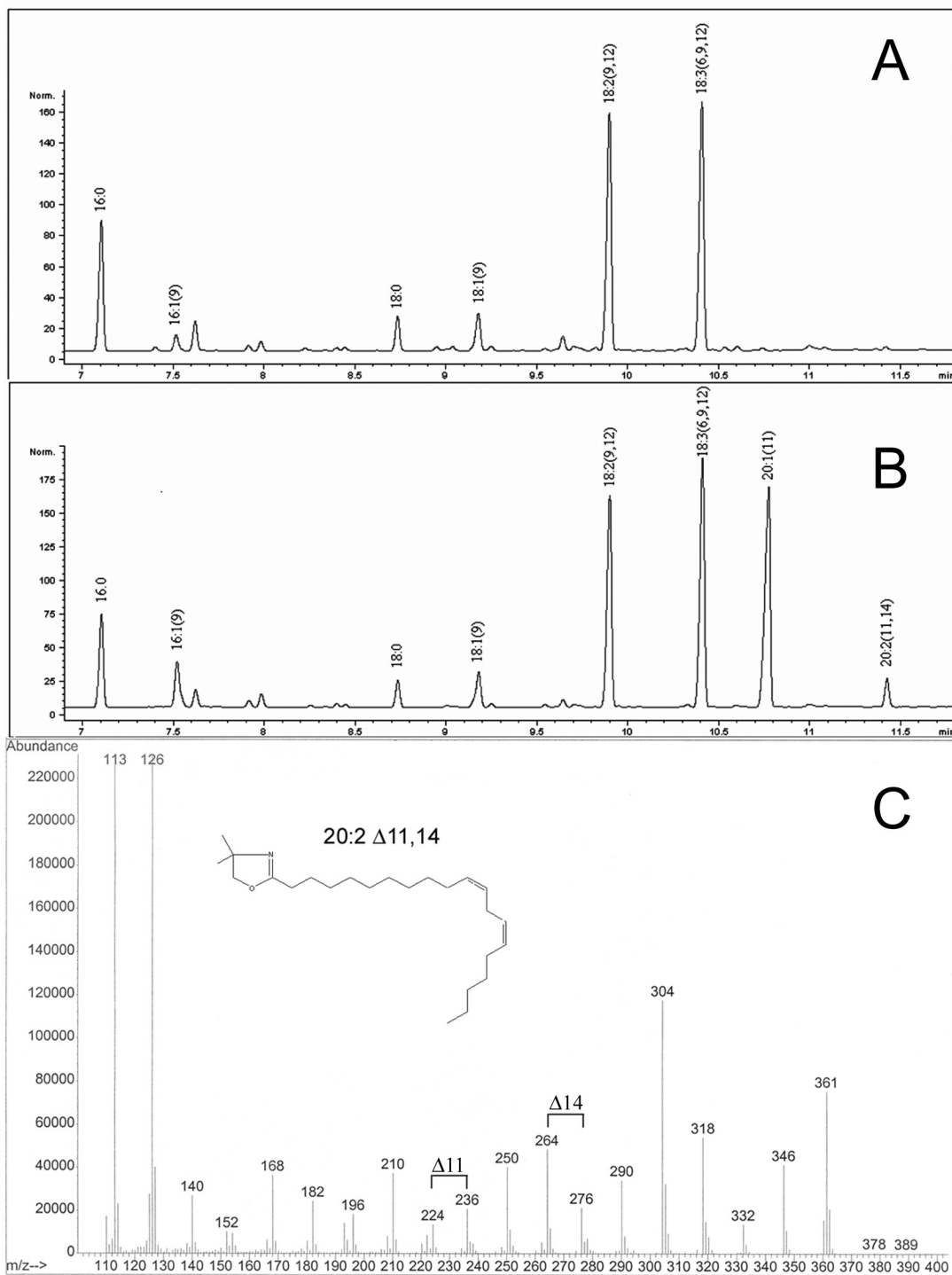
Figure 11. Verification of delta14 activity in *Tetrahymena pyriformis.* GC trace of fatty acid methyl esters derived from *Tetrahymena pyriformis* cells grown in media lacking exogenous fatty acids (A) and in media containing 20:1 as a free fatty acid (B). (C) GC-MS trace of the DMOX derivative of the fatty acid identified as 20:2(11,14).

```
                 1                                                               80
   At FAD2    (1) -------MGAGGRMPVPTSSKKSETDTTKRVPCEK---PPFSVGDLKKAIPPHCFKRSIPRSFSYLISDIIIASCFYYVA
   Ma del12   (1) MAPPNTIDAGLTQRHITTAAPTSAKPAFERNYQL---PEFTIKEIRECIPAHCFERSGLRGLCHVAIDLTWASLLFLAA
   Ce FAT-2   (1) ---------------MTIATKVNTNKKDLDTIKVP--ELPSVAAVKAAIPEHCFVKDPLTSISYLIKDYVLLAGLYFAV
   Tp TDM-1   (1) --------------MSNLSAKKSDTQTAIANDTVFNGEKEVSVGDIRKFIEAEYFVKRESRFLISVLFSLSLTLFTGFLA

                 81                                                              160
   At FAD2   (71) TNYFSLLPQPLSYLAWPLYWACQGCVLTGIWVIAHECGHHAFSDYQWLDDTVGLIFHSFLLVPYFSWKYSHRRHHSNTGS
   Ma del12  (78) TQIDKFENPLIRYLAWPAYWIMQGIVCTGIWVLAHECGHQSFSTSKTLNNTVGWILHSMLLVPYHSWRISHSKHHKATGH
   Ce FAT-2  (63) P----YIEHYLGWIGLLGWYWAMGIVGSALFCVGHDCGHGSFSDYEWLNDLCGHLAHAPILAPFWPWQKSHRQHHQYTSH
   Tp TDM-1  (67) DRYLRLEWAYFP--LWVAYAVIQGTIATGLWVLGHECGHKAFSDSDLINDALGYILHTPLLVPYFSWQWSHHVHHSKCNH

                 161                                                             240
   At FAD2  (151) LERDEVFVPKQKSAIKWYGK--------Y--------LNNPLGRIMMLTVQFVLGWELYLAFNVSGRPYDGFACHFFPNA
   Ma del12 (158) MTKDQVFVPKTRSQVGLPPKESAAAAVQEEDMSVHLDEEAPIVTLFWMVIQFLFGWEAYLIMNASGQDYGRWTSHEHTYS
   Ce FAT-2 (139) VEKDKGHPWVTEEDYNNRTA-------------------IEKYFAVIPISGWLRWNEIYTIVGLPDG------SHFWPWS
   Tp TDM-1 (145) LTEGETHVPRVESDPKVAMYIKLR-----------EIIGVESFSIWQILNILIIGWEAYLIFGATGGPARGFTSHEIVPN

                 241                                                             320
   At FAD2  (215) PIYNDRERLQIYLSDAGILAVCFGLYRYAAAQGMASMICLYGVPLLIVNAFLVLITYLQHTHPSLPHYDSSEWDWLRGAL
   Ma del12 (238) PIFEPRNFFDIIISDLGVLAALGALIYASMQLSLLTVTKYYIIPYLFVNFWLVLITFLQHTDPKLPHYREGAWNFQRGAL
   Ce FAT-2 (194) RLFETTEDRVKCAVSGVACAICAYIAFVLCDYSVYTFVKYYIPLLFQGLILIVITYLQHQNEDIEVYEADEWGFVRGQT
   Tp TDM-1 (214) KLFTKEKLVKVHLSNLGLIIVGYFLYLWAKNTSFTYVFAMYFGPYLIVNCWLTGITFLQHTEDTVPHYDASAWSWLRGAL

                 321                                                             400
   At FAD2  (295) ATVDRDYG-ILNKVFHNITDTHVAHHLFS-TMPHYNAMEATKAIKPILGDYYQFDGTPWYVAMYREAKECIYVEPDREGD
   Ma del12 (318) CTVDRSFGKFLDHMFHGIVHTHVAHHLFS-QMPFYHAEEATYHLKKLLGEYYVYDPSPIVVAVWRSFRECRFVEDHGD--
   Ce FAT-2 (274) QLIDRHWGFGLDNIMHNITNGIVHHFFTKIPHYHLLEATPAIKKAIEPLKDTQYGYKREVNYNWFFKYLHYNVTLDYL
   Tp TDM-1 (294) CLIDRNYPEFINALHFDIGSTHVVHEIFH-EMPHYSAREANIYLKQILGPAYIKDKKSITKAAFDAGKLACVSEKEKG--

                 401             423
   At FAD2  (373) ---KKGVYWYNNKL---------
   Ma del12 (395) ------VVFFKK-----------
   Ce FAT-2 (354) THKAKGVLQYRSGVEAAKAKKAQ
   Tp TDM-1 (371) ------FYYWK------------
```
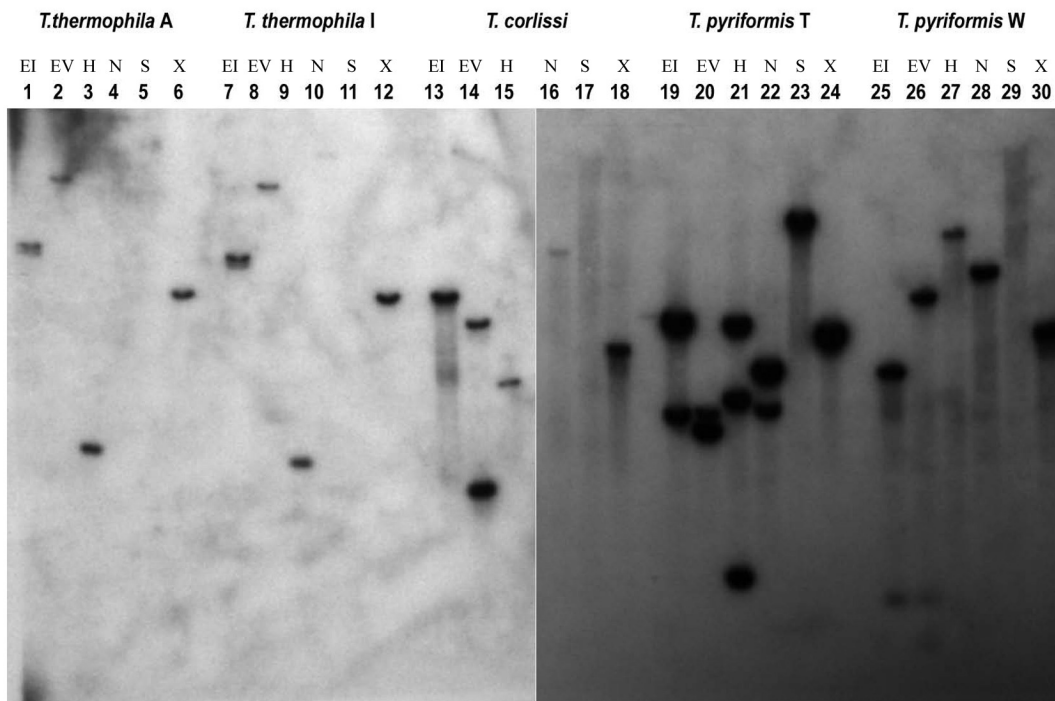
Figure 12. The delta 12 desaturase homolog, TDM-1, aligns with delta 12 desaturases from *Arabidopsis thaliana* (At FAD2), *Mortierella alpina* (Ma del12), and *Caenorhabditis elegans* (Ce FAT-2). TDM-1, and therefore the Tt del12, align with known delta 12 desaturases. Areas of highest conservation are around the three histidine boxes which are underlined. Conserved residues are boxed in gray.

	T.thermophila A						T. thermophila I						T. corlissi						T. pyriformis T						T. pyriformis W					
EI	EV	H	N	S	X		EI	EV	H	N	S	X	EI	EV	H	N	S	X	EI	EV	H	N	S	X	EI	EV	H	N	S	X
1	2	3	4	5	6		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

**Figure 13.** Southern Blot for delta12 homologs in *Tetrahymena* genomic DNA. DNA from *T. thermophila* A and I, *T. corlissi*, and *T. pyriformis* T and W was isolated and digested with EcoRI (EI), EcoRV (EV), HindIII (H), NdeI (N), SalI (S) or XbaI (X) and electrophoresed. Following blotting and UV cross-linking membranes were probed with p32 radiolabeled tdm-1 cDNA. Banding pattern was indicative of only one delta 12 homolog.
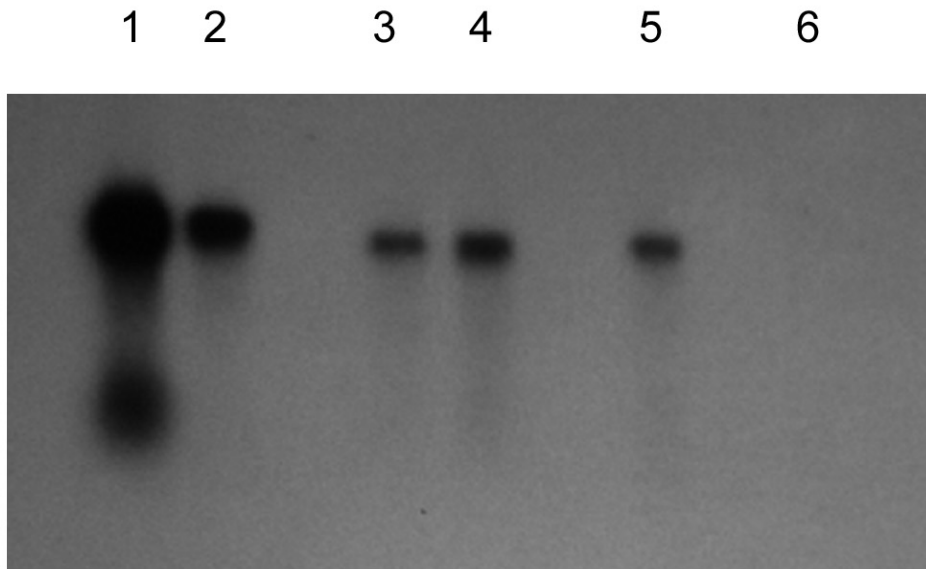
Figure 14. Northern Blot of *T. pyriformis* delta12 mRNA in yeast. RNA was trizol isolated from yeast transformed with the *T. pyriformis* delta12 cDNA in pYES2 and grown in galactose (lanes 1+2), tdm-1 in p416-GPD (lanes 3+4), tdm-1 in p416-TEF (lane 5) and the pYES2 empty vector grown in galadctose (lane 6). Blot was probed with p32 labeled tdm-1.
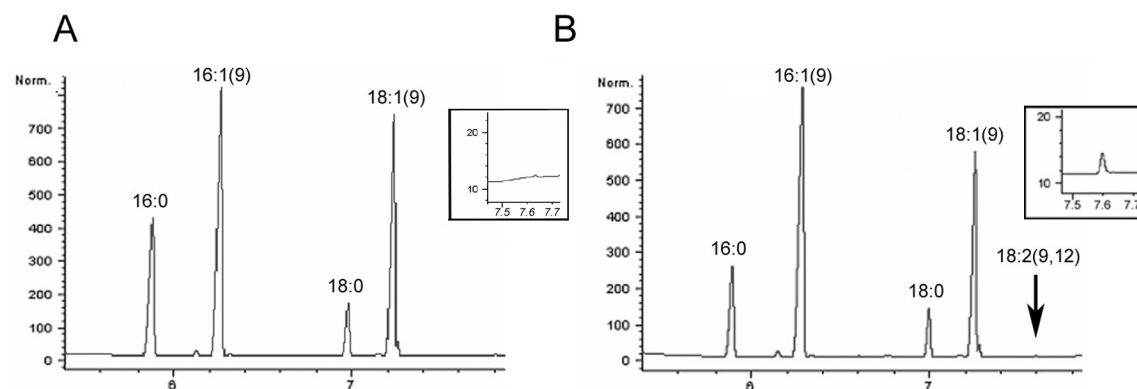
Figure 15. Delta12 desaturation with codon optimization of *T. thermophila* cDNA. Fatty acid methyl esters of yeast transformed with the codon optimized delta12 desaturase of *T. thermophila* in pYES2 were grown in media containing (A) glucose or (B) galactose and analyzed by gas chromatography. Desaturation of 18:1(9) to 18:2(9,12) is seen only with transgene induction by galactose. Insets with magnification of the lack of the delta 12 peak in glucos grown cells and the presence of a peak in the cells where expression of the desaturase was induced by galactose.
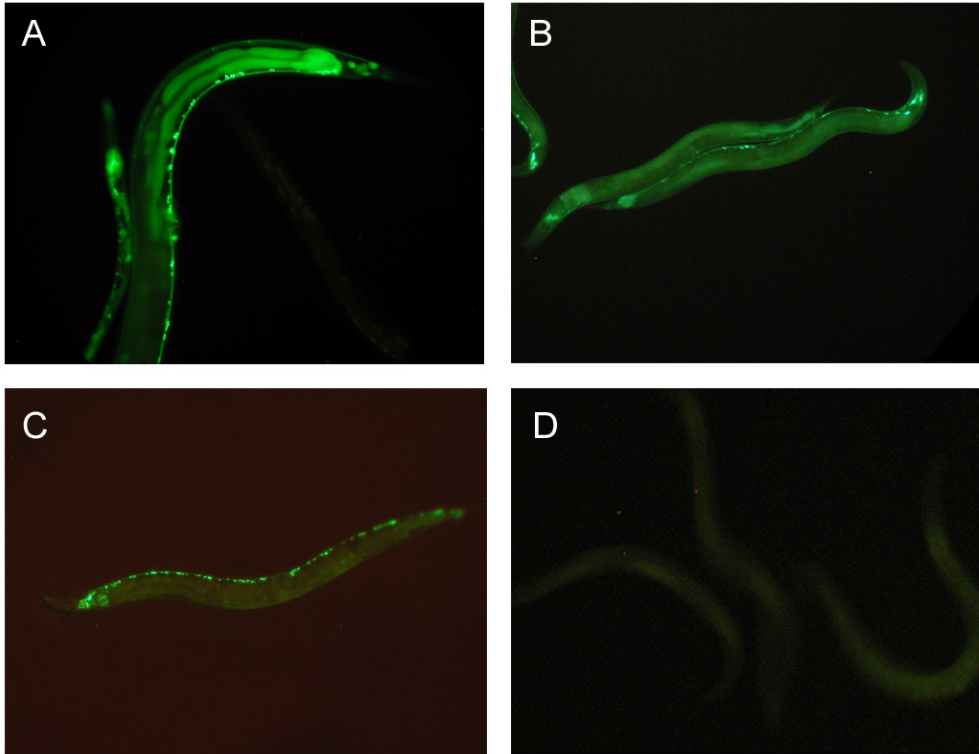
Figure 16. Expression of the TDM-1/GFP fusion protein in C. elegans. A fusion construct of was generated expressing a fusion of GFP and the T. pyriformis delta 12 desaturase with the C. elegans fat-1 promoter. Three independent transformation lines show GFP expression in the intestine and neurons (A), intestine (B) and neurons (C), and untransformed control worms have no GFP fluorescence (D).

CHAPTER FOUR

ALTERATION IN ARABIDOPSIS SEEDS LIPIDS BY THE EXPRESSION OF THE

DELTA8 DESATURASE OF *E. GRACILIS* AND THE 16:0 DESATURASE

OF *C. ELEGANS*

Introduction

Modification of the fatty acids of plant seed oils has been a major topic of study

for many years both because of the importance of fatty acids in human health and disease

and for their use in commercial applications. An essential tool for altering the

endogenous lipids of seed oils is the expression of fatty acid modification enzymes such

as thioesterases, desaturases, and hydroxylases. (Broun and Somerville, 1997; Eccleston

and Ohlrogge, 1998) Transformation of plants with enzymes that alter levels of

endogenous fatty acids or generate novel fatty acids has resulted in various levels of

success. Rarely, if ever, is the level of heterologously produced fatty acid as high as can

be found in the host plant; there are many known factors that could limit the production

of the novel fatty acid and ,undoubtedly, many unknown factors. At high levels of

transgene expression and enzyme action, activities of other enzymes may limit the

incorporation of the novel fatty acid into the triacylglyceride (TAG) pool. With the

heterologous expression of the *Umbellularia californica* (California Bay) lauryl-ACP

thioesterase in rapeseed accumulation of high levels of laurate (12:0) induced β-

oxidation. (Eccleston and Ohlrogge, 1998) Interactions with other key players like

cytochrome b_5, could also be a problem as could availability of substrates. While the

production of novel fatty acids by transgene expression is a somewhat hit and miss

process, desaturases and other fatty acid modification enzymes with high activity in oilseeds are much sought after.

Lipids of *Arabidopsis thaliana* seed have two pools of fatty acids that are very attractive as targets for modification by transgene expression. These are 16:0, the sixteen carbon saturated fatty acid, which makes up around 10% of total seed lipids, and 20:1, the monounsaturated twenty carbon fatty acid which is about 15% of the total oil in seeds. Interest in 20:1 focuses on its possible role as a substrate for desaturation to 20:5, EPA. EPA is a polyunsaturated $\omega 3$ fatty acid, which, along with docosahexaenoic acid (DHA, 22:6), is a key player in regulation of the inflammatory response in humans. As explained in Chapter Three, the $\Delta 12$ desaturase homolog was cloned from *Tetrahymena* species with the intent of using its activity in desaturating 20:1(11) to 20:2(11,14). 20:2 was not the intended final product, but a possible intermediate step in desaturating 20:1 to 20:5(5,8,11,14,17) by expressing four transgenes. The three other transgenes needed for this are the $\Delta 5$ desaturase of *C. elegans*, the $\Delta 8$ desaturase of *E. gracilis*, and the $\omega 3$ desaturase of *C. elegans*. To transform four different desaturase transgenes into *Arabidopsis*, a plant transformation vector was designed to express both the $\Delta 5$ and the $\Delta 8$ desaturase while another vector would express the $\Delta 14$ and $\omega 3$ desaturases. This strategy was adopted because if four desaturase cDNAs were cloned into four different vectors it would require four different selectable markers. With four separate transgenes then the occurrence of a plant in the T_2 generation that was homozygous for all four insertion sites in a randomly segregating population would be about $(\frac{1}{4})^4$, or $\frac{1}{256}$. This would require planting 750 or so seeds from each T_1 plant to have a reasonable probability of getting a single homozygous T_2 plant. With the two double vectors the

average occurrence of a homozygous seed in the T_2 generation would now be $^1/_{16}$, a much more reasonable number. This chapter will describe the generation of the $\Delta5/\Delta8$ double vector and its transformation into *Arabidopsis*. Seeds expressing this desaturase had an altered fatty acid profile with the desaturation of 20:1(11) to 20:2(8,11) and of 20:2(11,14) to 20:3(8,11,14).

While we were looking at utilizing 20:1 at a substrate for desaturation to a polyunsaturated fatty acid, we were also interested in the desaturation of 16:0. The majority of saturated fatty acids in *Arabidopsis* seeds are 16:0, which is also a component of many commercial vegetable oils. Health professionals recommend a diet low in saturated fat, so the reduction of the amount of saturated fat in oilseeds offers the potential for a major human health benefit. In *Arabidopsis* seeds, most saturated 16:0 generated in the plastids has two possible fates. It can be elongated to 18:0, which is desaturated to 18:1, 18:2 and 18:3, or it can be released from ACP by a thioesterase and exported to the cytoplasm for incorporation into the acyl-CoA pool, the extraplastydal glycerolipids, and TAGs. Both the elongase responsible for elongation of 16:0 to 18:0 (3-ketoacyl-ACP-synthase II) and the thioesterase responsible for release of the 16:0 into the cytosol have been targets for genetic engineering to reduce 16:0 in castor seeds. In *Arabidopsis* seeds, once the 16:0 has reached the cytosol, there is no native desaturase that can act on the fatty acid, which is present as an acyl-CoA moity. But there are acyl-CoA desaturases known to act on 16-carbon saturated fatty acids. One such desaturase is FAT-5 from *C. elegans*.

C. elegans has a complicated fatty acid desaturation pathway with many desaturases, including three $\Delta9$ deaturases FAT-5, FAT-6 and FAT-7. When expressed

in *ole1* yeast (a knockout of the Δ9 deaturase) these could all rescue the fatty acid

dependent phenotype of the yeast strain. (Watts and Browse, 2000) However they had

different substrate preferences. FAT-6 and FAT-7 could desaturate 16:0 to 16:1, but

preferred 18:0 as a substrate. In contrast, FAT-5 readily desaturated 16:0 to 16:1, but had

almost no activity on 18:0. Since FAT-5 has activity almost exclusively on 16:0, it was

an ideal enzyme to act on the pool of 16:0-CoA in developing oil seeds. Seeds from

plants transformed with a vector containing fat-5 under the control of the seed-specific

phaseolin promoter were examined for activity of the desaturase in desaturation of 16:0

to 16:1. The lines looked at had a wide range in percentages of 16:0 and 16:1, some lines

had a reduction in 16:0 from 10% to close to 3%.

Materials And Methods

Generation of the delta 5 delta 8 double vector

Primers were designed to the 5' and 3' ends of the cDNAs of the Δ5 desaturase of *C. elegans* and the Δ 8 desaturase of *E. gracilis*. Primers for the amplification of the Δ5 were 5'del5BaXh (5' GCCGGATCCCTCGAGATGGTAT TACGAGAGCAAGAGCAT 3') and 3'del5HiNc (5'GCCCCATGGAAGCTTCTAGG CAATCTTTTTAGTCAA 3') and primers for the amplification of the Δ 8 were 5'del8AsKp (5' CCGGCGCGCCGGTACCATGAAGTCAAAGCGCCAAGC 3') and 3'del8BaXh (5' CCCTCGAGGGATCCTTATAGAGCCTTCCCCGCGGGTT 3'). PCR fragments were ligated into pCR-script Amp and used to electroporate DH10B *E. coli*. Transformed cells were isolated by selective plating and used to start 3ml liquid cultures. Plasmids were isolated from transformed *E. coli* with the Qiagen Mini-prep kit. The pCR-script containing the Δ5 was digested with XhoI and HindIII, and the pCR-script containing the Δ 8 was digested with Acc65I and BamHI. The restriction digests were electrophoresed on an agarose gel, and the fragments corresponding to the Δ5 and Δ 8 desaturases were excised with the Qiagen Gel Extraction kit. The Δ5 desaturase cDNA was then cloned into the pPHAS vector that was also digested with XhoI and HindIII. The Δ 8 desaturase cDNA was cloned into pOEA2, which had been digested with BamHI and Acc65I. These vectors were then used to transform *E. coli*, and cultures of transformed cells were then mini-prepped and digested again, the Δ 8 in pOEA2 with EcoRI, and the Δ5 in pPHAS with NotI. The Δ5 in pPHAS digest was electrophoresed on an agarose gel, and the fragment containing the desaturase was excised. Following gel extraction, the Δ5 fragment and the Δ 8 in pOEA2 vector were treated with the Klenow

fragment of DNA PolI (New England Biolabs) to generate blunt ends. The Δ 8 pOEA2 fragment was then treated with CIP (New England Biolabs) prevent self-ligation. The Δ5 fragment and Δ 8 vector were reacted together with T4 ligase, overnight at 15 degrees. The ligation reaction was then used to electroporate electrocompetent DH10B E. coli. Vectors were verified by PCR. Amplifications were done with primers for the Δ5 and Δ 8 cDNAs to ensure that both were found in the vector, and with a forward Δ 8 primer and reverse Δ5 primer to ensure correct orientation and distance between cDNAs.

Arabidopsis transformation

The double vector was used to transform wild type *Arabidopsis* via the Floral Dip method. *Agrobacterium tumefaciens* strain GU3101 was transformed with the double vector by electroporation. After two days, a colony was selected and a 5ml overnight culture was used to inoculate a flask with 250 ml of 2xYT. This culture was grown for 36 hours under gentamicin and spectomycin selection at 25 C. The cells were then be harvested by centrifugation and re-suspended in inoculation medium of 5% sucrose and 0.05% Silwet L-77 (OSi Specialties Inc. Danbury CT) at a fixed OD_{600} of 0.6.

The aboveground portion of the plants were dipped in the inoculation solution for 5 seconds and then removed. The inoculated plants were then tented in plastic wrap for 12 hours. Plastic wrap was then removed and the plants were allowed to mature and produce seeds. These original transformed plants are referred to as the T_0 population. Seeds from these transformed plants, T1 seeds, were then planted on soil wetted with water containing 430μL/L Finale, the commercial brand of Basta herbicide. Hemizygous T_2 plants were grown T_3 seed was collected. The T3 seed was analyzed for

activity of the Δ 8 and Δ5 desaturases by GC. Seeds lipids were derivatized to FAMEs as previously described and analyzed on the GC.

Analysis of FAT-5 expressing seeds

T1 seeds of the transformation of Arabidopsis thaliana with fat-5 in pOEA2 were obtained from Barbara King. T1 seed was planted on soil containing 430 μL/L Finale and T1 plants that were resistant to the herbicide were grown and seed was collected. The activity of FAT-5 expression in Arabidopsis seeds was then analyzed by GC as previously described.

Results

Expression of the Δ8 desaturase of *E. gracilis* in *Arabidopsis* seeds

In order to produce eicosapentaenoic acid from the endogenous 20:1(11) found in *Arabidopsis thaliana* seeds, four desaturases would be required. The proposed Δ14 desaturase of *Tetrahymena* would be needed as well as Δ5 and Δ8 desaturases and an ω3 fatty acid desaturase (Figure 8B). To transform these desaturases into *Arabidopsis*, a scheme involving two double vectors was planned, and the construction of one of these vectors was undertaken using the Δ5 and Δ8 desaturases. Generation of the double vector began with cloning the Δ5 desaturase into the pPHAS vector containing the phaseolin promoter and the NOS terminator and cloning the Δ 8 desaturase into pOEA2, which also had the phaseolin promoter and NOS terminator. A NotI digest of the Δ5 vector excised a fragment containing the just the promoter, cDNA and terminator. This fragment was then cloned into the pOEA2 vector containing the Δ 8 desaturase cDNA. The overall double vector consisted of phaseolin::delta 8::NOS::phaseolin::delta 5::NOS between the left and right borders. This vector also contained the BAR gene allowing for screening using resistance to the herbicide Basta. This vector was used to transform *Agrobacterium tumefasciens* by electroporation. This *Agrobacterim* strain was then used to transform wild type *Arabidopsis thaliana* of the Columbia ecotype by the floral dip method. The innoculated T_0 plants were grown and their T_1 seeds were harvested. T_1 seeds were then planted on soil containing Basta and plants resistant to the herbicide were grown so that the fatty acids of the T_2 seeds could be analyzed.

Seeds from the primary transformants (T_2 seeds from T_1 plants) of the double vector were examined by methyl ester derivatization and gas chromatography. The traces

were examined for any changes in fatty acid profile. The seeds did have a change in fatty acid in the placement of the double bond in the 20:2 found in the seeds. (Figure 15) There is a small amount of endogenous 20:2(11,14) found in *Arabidopsis* seeds due to the elongation of 18:2(9,12), but in these transformed plants the majority of the 20:2 found had double bonds at the 8th and 11th carbons, as verified by mass spectrometry. (Figure 15C) This indicated the activity of a $\Delta 8$ desaturase acting on the endogenous 20:1(11). The activity of the $\Delta 8$ also seemed to decrease the endogenous amounts of 20:2(11,14). This could be partly due to substrate competition, and partly due to further desaturation of the endogenous 20:2(11,14) by the $\Delta 8$ desaturase. 20:3(8,11,14) was found in the double vector transformed plants but alone is not enough to account for the lack of 20:2(11,14) in these seeds. Evidence of the activity of the $\Delta 5$ desaturase was not seen in these seeds, however if there was a 20:3(8,11,14) or a 20:4(5,8,11,14) present, they would be in low enough amounts that they would be very difficult or impossible to detect with the methods that we employed.

Expression of the palmitoyl-CoA desaturase of *C. elegans* in *Arabidopsis* seeds

Plant oils contain far lower concentrations of saturated fatty acids than animal fats, but these oils still have significant amounts of saturated fats, in the range of 10-20% for commercial vegetable oils. In an approach to reduce the amount of saturated fatty acids found in *Arabidopsis* seeds, an acyl-CoA desaturase from *C. elegans* was expressed in plants. FAT-5 from *C. elegans* had been previously identified as an $\Delta 9$ acyl-CoA desaturase that acted specifically on 16:0. To express this desaturase in plants, the cDNA was first cloned into the plant expression vector pOEA2, containing the seed-specific promoter phaseolin. Plants were then transformed via *Agrobacterium* using floral dip.

Basta selection was used to identify T_1 seeds containing the BAR (Basta resistance) gene and therefore the desaturase transgene. The resistant T_1 plants were hemizygous, and therefore the T_2 seeds examined were a segregating population of homozygous for the $\Delta 9$, hemizygous, and homozygous wild type.

The fatty acid profile of T_2 seeds from the T_1 plants were analyzed using gas chromatography. Seeds were boiled in methanol containing sulfuric acid to generate the fatty acid methyl ester derivative of the fatty acid. The FAMEs were separated by GC and the traces were examined for changes in the fatty acid profile. In wild type *Arabidopsis thaliana* seeds the percent of total fatty acids found as 16:0 is about 10% and the amount of 16:1(9) is less than 0.5%. In the 60 T_2 seed lines examined some lines had a fatty acid trace very similar to that of wild type, but some lines had amounts of 16:1 between 6 and 7%, and these high amounts corresponded to comparable decreases in the amount of 16:0. This meant that in some lines the % of 16:0 was as low 3%.

Discussion

In addition to the modification of yeast lipids by heterologous fatty acid desaturase expression, desaturases were also expressed in *Arabidopsis* to increase the amount of a desired fatty acid (20:2(8,11)), and decrease the amount of an undesirable one (16:0) in seed lipids. Chapter Three dealt with the attempt to heterologously express the *Tetrahymena* protein responsible for Δ14 desaturation, but this was just one of four desaturases that would be needed to make eicosapentaenoic acid (EPA, 20:5) in *Arabidopsis* seeds. Three other desaturases would also have been expressed in plants. Two of these were the Δ 8 desaturase of *Euglena gracilis* and the Δ5 desaturase of *Caenorhabditis elegans.* To this end, the cDNAs encoding these proteins were cloned into a double vector and used to transform *Arabidopsis*. When seeds of transformed plants were identified, it was found that the fatty acids were altered. When the fatty acids of transformed seeds were examined, there were two fatty acid peaks not seen in wild type seeds. The twenty-carbon fatty acids found in wild type *Arabidopsis* seeds are 20:0, 20:1(11), 20:1(13) and 20:2(11,14). These fatty acids are all elongation products of 18 carbon fatty acids, 18:0, 18:1(9), 18:1(11), and 18:2(9,12) respectively. In seeds containing the Δ8 and Δ5 transgenes, there was still 20:0, 20:1(11), 20:1(13) and a very small amount of 20:2(11,14), but there was also a new 20-carbon fatty acid with two double bonds and the presence of a 20-carbon fatty acid with three double bonds. The novel 20:2 fatty acid had a shorter retention time than 20:2(11,14), and, with the GC column used for separation, this indicated that the new fatty acid had a double bond closer to the carboxyl end of the desaturase. This could be assumed to be the Δ8

desaturation product of 20:1(11) which is 20:2(8,11). The Δ8 desaturase of *Euglena*

gracilis had been previously expressed in *Arabidopsis* leaves, and shown to act on

20:2(11,14) and 20:3(11,14,17), but had not been used to modify the endogenous pool of

20:1 found in *Arabidopsis* seeds. (Qi et al., 2004) When the desaturation activity of the

Δ8 of *Euglena* was examined by yeast expression, the desaturase converted 6.1 % of the

fed 20:1(11) to 20:2(8,11). (Wallis and Browse, 1999) In these *Arabidopsis* seeds, we

see an increase in the amount of desaturation as compared with yeast, with 10.1% of

20:1(11) desaturated to 20:2(8,11). The desaturase acting on the 20:1 was believed to be

the Δ8 and not the Δ5 desaturase which did not desaturate 20:1 to 20:2(5,11) in yeast.

(Watts and Browse, 1999) For verification, the identity of the fatty acid was determined

by mass spectrometry of the DMOX derivative to be 20:2(8,11). The Δ8 desaturase was

also responsible for the presence of a 20:3 fatty acid, which was identified by mass

spectrometry to be 20:3(8,11,14), a desaturation product of 20:2(11,14). It is likely that

this desaturation is largely responsible for the lack of 20:2(11,14) found in seeds

expressing the Δ8 transgene. In yeast it was shown that the Δ8 desaturase of *Euglena*

will desaturate 14.8% of fed 20:2(11,14), in these seeds it appears that a greater amount

of endogenous 20:2 is desaturated to 20:3. In *Arabidopsis* seeds of the Columbia wild

type, 20:2(11,14) is about 2% of the total fatty acids, and in the seeds of transformed

plants the amount of 20:2(11,14) is less that 1%, and the 20:3(8,11,14) is about 0.95% of

the total fatty acids. It is known from studies of the Δ5 desaturase in yeast, that it will

desaturate 55% of fed 20:3(8,11,14) to 20:4(5,8,11,14) but there is no data available

about the desaturation of 20:2(8,11) to 20:3(5,8,11). Due to the variety of substrates this

desaturase can use, it would be likely that 20:2(8,11) would be a usable substrate.

However there was no peak that was identified as 20:3(5,8,11), the desaturation product if both the Δ8 and Δ5 desaturases acted upon 20:1(11). It is true that there is only a small pool of 20:2(8,11) available for desaturation, but it is surprising that a Δ5 desaturation peak was not seen. When fatty acids were derivatized and analyzed by GC-MS, 20:3(5,8,11) was never identified. With this the ability of the Δ8 desaturase to act on the endogenous 20-carbon fatty acids of Arabidopsis seeds was verified, but products of desaturation by the Δ5 desaturase were not identified. So whether or not the *C. elegans* Δ5 desaturase is present and active in seeds is unknown.

The seed lipids of *Arabidopsis thaliana* were not only altered to produce a novel fatty acid, but also to decrease the amount of saturated fatty acid found in these seeds. The majority of saturated fatty acids in Arabidopsis seeds is 16:0, and this molecule makes up 8-10% of the total seed fatty acids. To decrease the amount of saturated fat, a Δ9 desaturase from *C. elegans* specific to 16-carbon fatty acids was transformed into *Arabidopsis* under the control of the seed-specific phaseolin promoter. In oil seeds, the majority of the fatty acids that are exported to the cytosol (95%) are in the form of 16:0 or 18:1. Once in the cytosol, these fatty acids can be part of the acyl-CoA pool or be incorporated into the extraplastidyl glycerolipids. As components of the glycerolipids, 18:1 can be further desaturated by FAD2 (fatty acid desaturase 2, a Δ12) and FAD3 (an omega 3) to 18:2(9,12) and 18:3(9,12,15), or it can be elongated to 20:1 by FAE. But the 16:0 cannot be modified by these enzymes and will therefore be incorporated into the triacylglycerols without desaturation. It is this lack of a 16:0 desaturase in the cytosol of *Arabidopsis* seeds that was addressed with the transformation of *Arabidopsis* with FAT-5. FAT-5 is a membrane bound fatty acid desaturase that can act to desaturase 16:0

found in the acyl-CoA pool in the ER of seeds. When this Δ9 desaturase was used to transform Arabidopsis under the control of the seed-specific phaseolin promoter, a change in the amounts of 16:0 and 16:1 was found in plants containing the transgene. (Figure 16) Seventy-four lines of T_2 seeds (the segregating seeds of the hemizygous T_1 plants) were examined by GC to examine levels of desaturation in the 16-carbon fatty acids. While many lines had profiles very similar to wild-type seeds, with around 9% 16:0 and less that 1% 16:1(9), other lines had levels of 16:0 and 16:1 that were almost equal. In each case, the increase in 16:1 was the result of a decrease in the amount of 16:0. The lowering of the amount of 16:0 did not cause an increase in the export of 16:0 from the plastids as could be seen by a lack of change in the overall percentage of 16 carbon fatty acids in *Arabidopsis* seeds, it remained at a fixed percentage of 8-10%. This maintenance of a fixed percentage of 16 carbon fatty acids can be seen in the line with the highest desaturation seen (line 1), where even though the amount of 16:1 is increased to 6.6%, the overall percentage of 16-carbon fatty acids is only 10.1. (figure 16) With this line we see a 65% reduction of saturated 16:0 in the seeds.
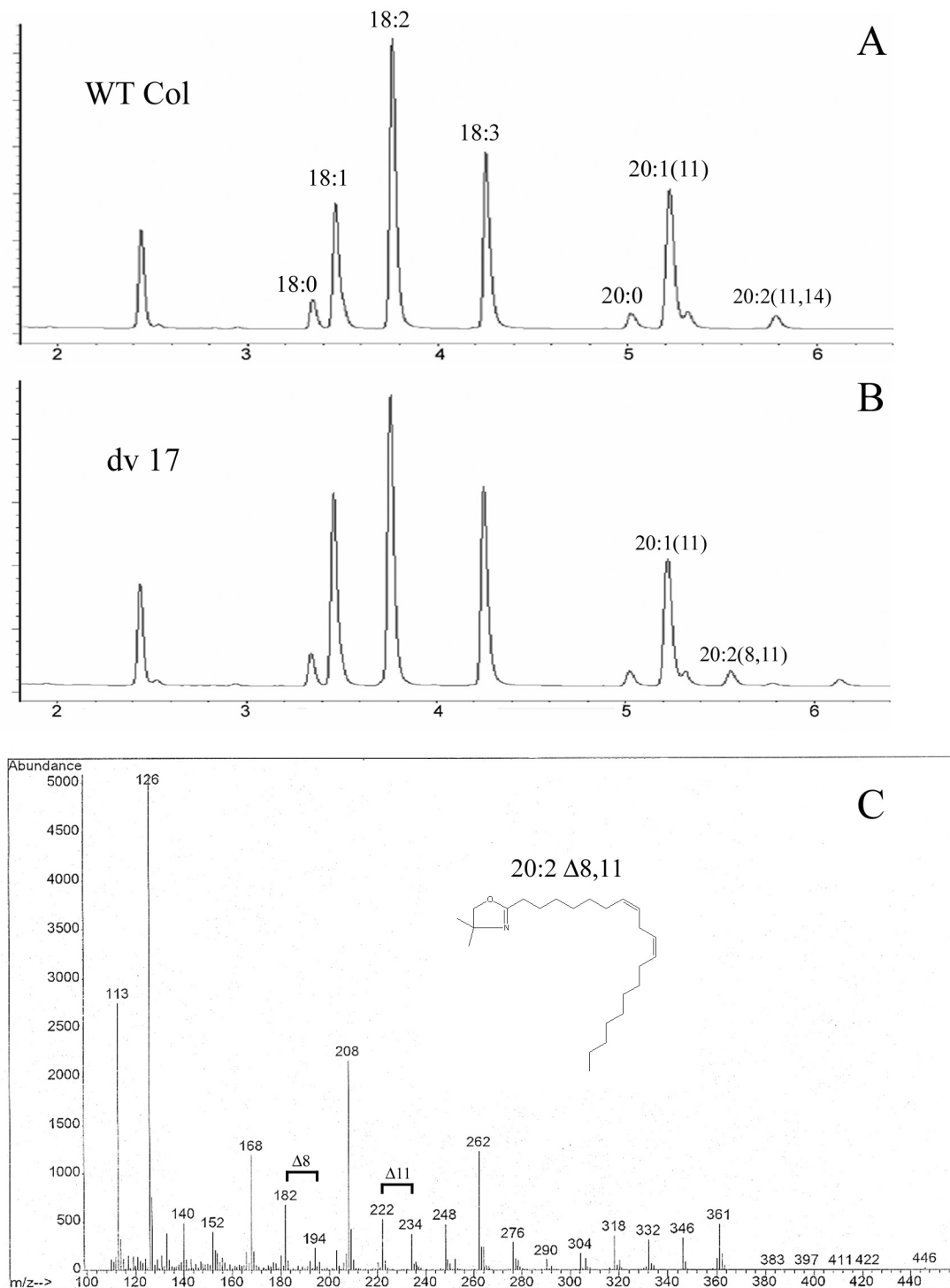
Figure 17. Production of 20:2(8,11) in seeds of double vector transformed *Arabidopsis*. Seeds of WT Columbia *Arabidopsis* and dv 17 seeds (T2 seeds of *Arabidopsis* transformed with a double vector containing the delta 5 desaturase of *C. elegans* and the delta 8 desaturase of *E. gracilis)* were derivatized to their fatty acid methyl ester and analyzed by gas chromatography. FAMEs of WT seeds (A) contained the 20-carbon fatty acids 20:0, 20:1(11) and 20:2(11,14). Seeds of double vector transformed *Arabidopsis* (B) also contained the delta 8 desaturation product 20:2(8,11). The identity of 20:2(8,11) was verified by GC-MS of the DMOX derivative of the FAME.
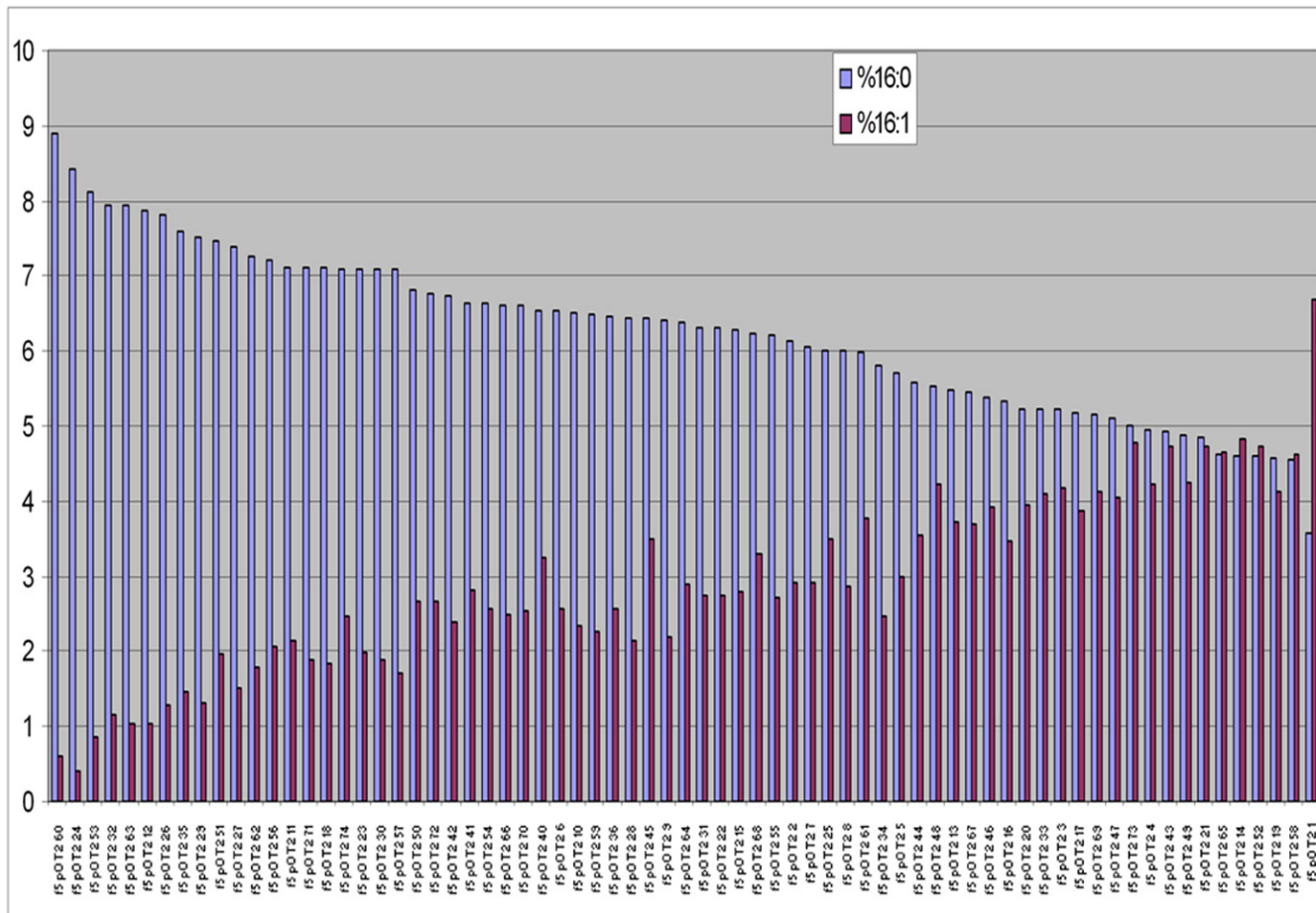
Figure 18. Variation of delta 9 desaturase in seeds of *Arabidopsis* transformed with FAT-5. *Arabidopsis* was transformed with the FAT-5 delta 9 desaturase of *C. elegans* in the pOEA2 vector with the seed specific phaseolin promoter. Seeds of T2 plants were analyzed for desaturation of 16:0 to 16:0. Fatty acids are graphed as the percentage of total fatty acids found as 16:0 or as 16:1.

Anggard E, Samuelsson B (1965) Biosynthesis of prostaglandins from arachidonic acid in guinea pig lung. Prostaglandins and related factors. 38. J Biol Chem **240:** 3518-3521

Avelange-Macherel MH, Macherel D, Wada H, Murata N (1995) Site-directed mutagenesis of histidine residues in the delta 12 acyl-lipid desaturase of Synechocystis. FEBS Lett **361:** 111-114

Babcock T, Helton W, Espat J (2000) Eicosapentaenoic Acid (EPA): An Anti-inflammatory -3 Fat With Potential Clinical Applications. Nutrition **16:** 1118-1200

Beck SA, Smith KL, Tisdale MJ (1991) Anticachectic and antitumor effect of eicosapentaenoic acid and its effect on protein turnover. Cancer Res **51:** 6089-6093

Broun P, Somerville C (1997) Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic Arabidopsis plants that express a fatty acyl hydroxylase cDNA from castor bean. Plant Physiol **113:** 933-942

Browse J, McConn M, James D, Jr., Miquel M (1993) Mutants of Arabidopsis deficient in the synthesis of alpha-linolenate. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. J Biol Chem **268:** 16345-16351

Cassel D, Ragona D, Carriero L, Kempe J, Conner R (1981) Metabolism of odd-numbered, normal fatty acids in Tetrahymena pyriformis W. Biochimica et Biophysica Acta **663:** 121-133

Conconi A, Smerdon MJ, Howe GA, Ryan CA (1996) The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. Nature **383:** 826-829

Connor W (2000) Importance of n-3 fatty acids in health and disease. American Journal of Clinical Nutrition **71S:** 171S-175S

Dailey HA, Strittmatter P (1980) Characterization of the interaction of amphipathic cytochrome b5 with stearyl coenzyme A desaturase and NADPH:cytochrome P-450 reductase. J Biol Chem **255:** 5184-5189

Dallerac R, Labeur C, Jallon JM, Knipple DC, Roelofs WL, Wicker-Thomas C (2000) A delta 9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in Drosophila melanogaster. Proc Natl Acad Sci U S A **97:** 9449-9454

Domergue F, Abbadi A, Heinz E (2005) Relief for fish stocks: oceanic fatty acids in transgenic oilseeds. Trends Plant Sci **10:** 112-116

Eccleston VS, Ohlrogge JB (1998) Expression of lauroyl-acyl carrier protein thioesterase in brassica napus seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. Plant Cell **10:** 613-622

Fukuchi-Mizutani M, Tasaka Y, Tanaka Y, Ashikari T, Kusumi T, Murata N (1998) Characterization of delta 9 acyl-lipid desaturase homologues from Arabidopsis thaliana. Plant Cell Physiol **39:** 247-253

Fukushima H, Martin C, Iida H, Kitajima Y, Thompson G, Jr. (1976) Changes in membrane lipid composition during temperature adaptation by a thermotolerant strain of Tetrahymena pyriformis. Biochimica et Biophysica Acta **431:** 165-179

Fukushima H, Nagao S, Okano Y, Nozawa Y (1977) Studies on Tetrahymena membranes. Palmitoyl-coenzyme a desaturase, a possible key enzyme for temperature adaptation in Tetrahymena microsomes. Biochimica et Biophysica Acta **488:** 442-453

Guillou H, D'Andrea S, Rioux V, Barnouin R, Dalaine S, Pedrono F, Jan S, Legrand P (2004) Distinct roles of endoplasmic reticulum cytochrome b5 and fused cytochrome b5-like domain for rat Delta6-desaturase activity. J Lipid Res **45:** 32-40

Hammarstrom S (1980) Leukotriene C5: a slow reacting substance derived from eicosapentaenoic acid. J Biol Chem **255:** 7093-7094

Helftenbein E (1985) Nucleotide sequence of a macronuclear DNA molecule coding for alpha-tubulin from the ciliate Stylonychia lemnae. Special codon usage: TAA is not a translation termination codon. Nucleic Acids Research **13:** 415-433

Hites RA, Foran JA, Schwager SJ, Knuth BA, Hamilton MC, Carpenter DO (2004) Global assessment of polybrominated diphenyl ethers in farmed and wild salmon. Environ Sci Technol **38:** 4945-4949

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:** 51-59

Horowitz S, Gorovsky M (1985) An unusual genetic code in nuclear genes of Tetrahymena. PNAS **82:** 2452-2455

Horrocks LA, Yeo YK (1999) Health benefits of docosahexaenoic acid (DHA). Pharmacol Res **40:** 211-225

Howe GA, Lightner J, Browse J, Ryan CA (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell **8:** 2067-2077

Huang YS, Chaudhary S, Thurmond JM, Bobik EG, Jr., Yuan L, Chan GM, Kirchner SJ, Mukerji P, Knutzon DS (1999) Cloning of delta12- and delta6-desaturases from Mortierella alpina and recombinant production of gamma-linolenic acid in Saccharomyces cerevisiae. Lipids **34:** 649-659

Hughes-Fulford M, Chen Y, Tjandrawinata RR (2001) Fatty acid regulates gene expression and growth of human prostate cancer PC-3 cells. Carcinogenesis **22:** 701-707

Kasai R, Kitajima Y, Martin C, Nozawa Y, Skriver L, Thompson G, Jr. (1976) Molecular control of membrane properties during temperature acclimation. Membrane fluidity regulation of fatty acid desaturase action? Biochemistry **15:** 5228-5233

Kitajima Y, Thompson G, Jr. (1977) Self-regulation of membrane fluidity. The effect of saturated normal and methoxy fatty acid supplementation on Tetrahymena membrane physical properties and lipid composition. Biochimica et Biophysica Acta **468:** 73-80

Kunst L, Taylor DC, Underhill EW (1992) Fatty acid elongation in developing seeds of Arabidopsis thaliana. Plant Physiol. Biochem. **30:** 425-434

Lederer F (1994) The cytochrome b5-fold: an adaptable module. Biochimie **76:** 674-692

Lees A, Korn E (1966) Metabolism of Unsaturated Fatty Acids in Protozoa. Biochemistry **5:** 1475-1481

Lightner J, Wu J, Browse J (1994) A Mutant of Arabidopsis with Increased Levels of Stearic Acid. Plant Physiol **106:** 1443-1451

Lo CJ, Chiu KC, Fu M, Lo R, Helton S (1999) Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF kappa B activity. J Surg Res **82:** 216-221

Martin C, Hiramitsu K, Kitajima Y, Nozawa Y, Skriver L, Thompson G (1976) Molecular control of membrane properties during temperature acclimation. Fatty acid desaturase regulation of membrane fluidity in acclimating Tetrahymena cells. Biochemistry **15:** 5218-5227

Michinaka Y, Aki T, Inagaki K, Higashimoto H, Shimada Y, Nakajima T, Shimauchi T, Ono K, Suzuki O (2001) Production of polyunsaturated fatty acids by genetic engineering of yeast. Journal of Oleo Science **3:** 359-365

Miquel M, Browse J (1992) Arabidopsis mutants deficient in polyunsaturated fatty acid synthesis. Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. J Biol Chem **267:** 1502-1509

Mitchell A, Martin C (1995) A novel cytochrome b5-like domain is linked to the carboxyl terminus of the Saccharomyces cerevisiae delta-9 fatty acid desaturase. Journal of Biological Chemistry **270:** 29766-29772

Nakashima S, Zhao Y, Nozawa Y (1996) Molecular cloning of delta 9 fatty acid desaturase from the protozoan *Tetrahymena thermophila* and its mRNA expression during thermal membrane adaptation. Biochemical Journal **317:** 29-34

Napier JA, Hey SJ, Lacey DJ, Shewry PR (1998) Identification of a Caenorhabditis elegans Delta6-fatty-acid-desaturase by heterologous expression in Saccharomyces cerevisiae. Biochem J **330 (Pt 2):** 611-614

Nozawa Y, Iida H, Fukushima H, Oki K, Onishi S (1974) Studies on Tetrahymena membranes: temperature-induced alterations in fatty acid composition of various membrane fractions in Tetrahymena pyriformis and its effect on membrane fluidity as inferred by spin-label study. Biochimica et Biophysica Acta **367:** 134-137

Nozawa Y, Kasai R (1978) Mechanism of thermal adaptation of membrane lipids in *Tetrahymena pyriformis* NT-1. Possible evidence for temperature-mediated induction of palmitoyl-CoA desaturase. Biochimica et Biophysica Acta **529:** 54-66

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J (1994) Arabidopsis FAD2 Gene Encodes the Enzyme That is Essential for Polyunsaturated Lipid Synthesis. Plant Cell **6:** 147-158

Peyou-Ndi M, Watts J, Browse J (2000) Identification and Characterization of an Animal Δ^{12} Fatty Acid Desaturase Gene by Heterologous Expression in *Saccharomyces cerevisiae*. Archives of Biochemistry and Biophysics **376:** 399-408

Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier J, Stobart A, Lazarus C (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nature Biotechnology **22:** 739-745

Sayanova O, Beaudoin F, Libisch B, Castel A, Shewry PR, Napier JA (2001) Mutagenesis and heterologous expression in yeast of a plant Delta6-fatty acid desaturase. J Exp Bot **52:** 1581-1585

Sayanova O, Shewry PR, Napier JA (1999) Histidine-41 of the cytochrome b5 domain of the borage delta6 fatty acid desaturase is essential for enzyme activity. Plant Physiol **121:** 641-646

Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA (1997) Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta6-desaturated fatty acids in transgenic tobacco. Proc Natl Acad Sci U S A **94:** 4211-4216

Shanklin J, Whittle E, Fox BG (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry **33:** 12787-12794

Simopoulos AP (2003) Essential fatty acids in health and chronic diseases. Forum Nutr **56:** 67-70

Spychalla JP, Kinney AJ, Browse J (1997) Identification of an animal omega-3 fatty acid desaturase by heterologous expression in Arabidopsis. Proc Natl Acad Sci U S A **94:** 1142-1147

Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A **97:** 10625-10630

Strittmatter P, Spatz L, Corcoran D, Rogers MJ, Setlow B, Redline R (1974) Purification and properties of rat liver microsomal stearyl coenzyme A desaturase. Proc Natl Acad Sci U S A **71:** 4565-4569

Stukey J, McDonough V, Martin C (1989) Isolation and characterization of OLE1, a gene affecting fatty acid desaturation from Saccharomyces cerevisiae. Journal of Biological Chemistry **264:** 16537-16544

Van D, Beerthuis RK, Nugteren DH, Vonkeman H (1964) The Biosynthesis of Prostaglandins. Biochim Biophys Acta **90:** 204-207

Vick BA, Zimmerman DC (1984) Biosynthesis of Jasmonic Acid by Several Plant Species. Plant Physiol **75:** 458-461

Wallis JG, Browse J (1999) The Delta8-desaturase of Euglena gracilis: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. Arch Biochem Biophys **365:** 307-316

Watts JL, Browse J (1999) Isolation and characterization of a Delta 5-fatty acid desaturase from Caenorhabditis elegans. Arch Biochem Biophys **362:** 175-182

Watts JL, Browse J (2000) A palmitoyl-CoA-specific delta9 fatty acid desaturase from Caenorhabditis elegans. Biochem Biophys Res Commun **272:** 263-269

Wu J, James DW, Jr., Dooner HK, Browse J (1994) A Mutant of Arabidopsis Deficient in the Elongation of Palmitic Acid. Plant Physiol **106:** 143-150

Yokoo EM, Valente JG, Grattan L, Schmidt SL, Platt I, Silbergeld EK (2003) Low level methylmercury exposure affects neuropsychological function in adults. Environ Health **2:** 8

Zhao Y, Nakashima S, Nozawa Y (1996) Molecular Cloning of a Gene (Tp9ds) Encoding Delta 9 Fatty Acid Desaturase and Growth-associated Changes in mRNA Expression in *Tetrahymena pyriformis*. European Journal of Protistology **32:** 539-544