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The Effect of 2*E*,4*E*-Decadienal on Lipid-Related Gene-Expression in *Phaeodactylum tricornutum*

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The Effect of 2*E*,4*E*-Decadienal on Lipid-Related Gene-Expression in *Phaeodactylum tricornutum*

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Report

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

The University of Texas at Austin

August 2013

Dedication

This report is dedicated to my husband, Jeremy Barras, whose love and support made it possible for me to complete this work.

Acknowledgements

I would like to acknowledge Dr. Mona Mehdy and Dr. K. Sathasivan for their support and guidance throughout this process. I would also like to acknowledge Tanya Sabharwal for giving so much of her time to help me learn molecular biology techniques and carry out my experiment. In addition, I would like to thank Tharindu Weeraratne, Nancy Wahl, and Hali Fechtmeyer for their assistance and encouragement during my time in the lab.

Finally, I would like to express my gratitude to Dr. Mary Walker and the rest of the staff at the UTeach Masters Program. This program and the professors that are a part of it helped make me a better teacher and I will always be grateful to them for doing so.

Abstract

The Effect of 2*E*,4*E*-Decadienal on Lipid-Related Gene-Expression in *Phaeodactylum tricornutum*

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Microalgae have been proposed as a potential feedstock for biofuel production, and as a result, interest in the biology of these organisms has intensified. These organisms also synthesize lipids that are vital to human health and nutrition. Stress has been shown to have an effect on lipid composition and gene expression in microalgae, but many studies have focused on the effects of abiotic stressors. The purpose of this study was to investigate the effect of biotic stress on lipid-related gene expression in *Phaeodactylum tricornutum,* a model species of microalgae. The source of biotic stress used in this study was 2*E*,4*E/Z*-decadienal, a diatom-derived oxylipin that has been shown to function as a stress signal among diatoms. Real-time RT-qPCR analysis revealed that expression of a patatin-like phospholipase was significantly decreased in decadienal-treated cultures as compared to a solvent control. The expression of a delta-9 desaturase gene believed to be responsible for production of 16:1 fatty acids was increased by a factor of 12. FabI, a gene involved in fatty acid biosynthesis, and PtD5a, which codes for an ER-localized desaturase, were both down-regulated in cells exposed to decadienal. However, changes

in expression were only shown to be significant for the patatin-like phospholipase gene. Increased expression of the delta-9 desaturase gene may be a protective mechanism against infection from pathogens, since 16:1 fatty acids have been shown to have antibacterial properties. Regulation of membrane desaturation may also serve to stabilize photosynthetic membranes during times of stress. The down-regulation of the phospholipase gene was surprising, since the release of fatty acids from membrane lipids for oxylipin production is a common response to stress. It is recommended that this experiment be improved upon and expanded in order to determine whether the results obtained are reproducible and how these changes in gene expression correlate with physiological effects.

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Chapter 1: Introduction

The use of fossil fuels to meet global energy and transportation needs is now seen as unsustainable, due to depleting resources and increases in atmospheric carbon levels that result from their use (NRC, 2010; Shafiee and Topal, 2009). The combustion of fossil fuels has led to increased amounts of greenhouse gases in Earth's atmosphere, and as a result, global climate change is threatening the stability of ecosystems, food production, and social welfare (IPCC, 2007). Biodiesel has been proposed as an alternative to petroleum-based products, but current biodiesel sources have limited ability to replace fossil fuels. Oil-rich crops such as corn, soybean, and canola have been used in biodiesel production, but these crops require large amounts of arable land (Chisti, 2007). In addition, use of these food crops for fuel production has the potential to affect global food prices (Sayre, 2010). Therefore, it is unlikely that these sources will ever replace petroleum-based fuels (Chisti, 2007).

Microalgae have been proposed as a source of biodiesel capable of meeting global demand for transport fuels (Chisti, 2007; Mata, Martins, and Caetano, 2009). Their high lipid content and rapid growth rate make them an attractive alternative to land-based systems (Mata et al., 2009; Sayre, 2010). In addition, microalgae require less area and have a much higher productivity than agricultural crops, reaching levels of 121,104 kg biodiesel/ha year in high-oil-content algae as compared to 4747 kg/ha year in the most productive plant source (Mata et al., 2009). Microalgae can also help mitigate CO_2 levels in the atmosphere, because they sequester CO_2 through photosynthesis (Sayre, 2010). As a result, interest in microalgae as a potential biofuel source has increased in recent years.

As a result of this interest, researchers have begun to elucidate the pathways responsible for lipid production in microalgae and develop techniques for increasing lipid

content in these organisms. In addition, molecular tools for the study of microalgae are currently being developed. The genomes of several species of microalgae have now been sequenced. In addition, expressed sequence tag (EST) databases and molecular tools required for functional genomics have been established for a number of species (Radakovits, Jinkerson, Darzins, and Posewitz, 2010). One species for which such tools have been developed is *Phaeodactylum tricornutum*, a member of the class Bacillariophyta (Bowler et al., 2008; Maheswari et al., 2010).

Bacillariophyta, also known as diatoms, are a class of photosynthetic algae that are found in freshwater and marine environments around the world (Bold and Wynne, 1978; Tiffany and Thomas, 1968). This diverse group of species generates about 20% of the organic carbon that is produced each year by photosynthesis, and is responsible for 40% of marine primary production (Brzezinski, Villareal, and Lipschultz, 1998; Nelson, et al., 1995). In addition to playing an important role in biogeochemical cycles and pelagic food webs, diatoms also produce many high-value lipids. The major lipids produced by *P. tricornutum* can be utilized for biofuel production, as previously mentioned, but are also important in human nutrition and health (Desbois, Mearns-Spragg, and Smith, 2008; Siriwardhana, Kalupahana, and Moustaid-Moussa, 2010). Therefore, it is important to understand the processes underlying lipid production in diatoms, and *P. tricornutum* can serve as a model species for studying these processes.

One commonly-researched strategy for increasing and controlling lipid production in microalgae is the application of environmental stress. Nutrient deprivation, for example, has been shown to increase lipid content in many species of microalgae (for review, see Sharma, Schumann, and Schenk, 2012). However, stress-induced changes in lipids do not follow one general rule. Alterations in lipid composition are condition- and species-specific. For example, Alonso, Belarbi, Fernandez-Sevilla, Rodriguez-Ruiz, and

Grima (2000) found that nitrogen deprivation led to increases in 16:0 and 16:1 fatty acids, and decreases in 16:2, 16:3 and 20:5 fatty acids in *P. tricornutum*. On the other hand, Liang, Beardall, and Heraud (2006) demonstrated that UV stress resulted in increases in polyunsaturated fatty acids (PUFAs) and decreases in saturated fatty acids (SFAs) in this species. Clearly, stress has an effect on lipid composition in microalgae, but these effects are complex in nature.

Most studies that have examined the effects of stress on lipid synthesis and composition in microalgae have focused on sources of abiotic stress (Sharma et al., 2012). Studies of biotic stressors and their effect on lipids have are not as abundant. Therefore, the aim of this study is to investigate the expression of genes involved in the synthesis, desaturation, and metabolism of fatty acids in *P. tricornutum* exposed to biotic stress. It is important to examine these processes in more detail because the ability to understand and control lipid-related gene expression is important if microalgae are to be used for large-scale production of valuable lipids.

The source of biotic stress used for this study was 2E,4E/Z-decadienal (decadienal, or DD). Decadienal is a diatom-derived polyunsaturated aldehyde (PUA) that has been shown to have a variety of effects on marine organisms. For example, copepods that graze on diatoms experience reduced reproductive success due to induced apoptosis in embryos, teratogenesis, and nauplii death (for review, see Leflaive and Ten-Hage, 2009). This impairment of reproduction has been tied to decadienal and other oxylipins produced by diatoms when they are grazed on (Miralto et al., 1999). Decadienal is considered a model oxylipin, and its negative effects on reproduction and growth have been demonstrated in many organisms including bacteria, crustaceans, echinoderms, and algae (Leflaive and Ten-Hage, 2009). In *P. tricornutum*, DD has been shown to trigger a stress response involving intercellular calcium and nitric oxide (NO) (Vardi et al., 2006;

Vardi et al., 2008). When *P. tricornutum* cells are exposed to high levels of DD, this NO-mediated stress response induces programmed cell death. However, Vardi et al. (2006) found that sub-lethal levels of DD could confer resistance to future aldehyde exposure, indicating that this aldehyde functions as an early warning signal, or "infochemical" among diatoms. Because other types of stress have been shown to elicit changes in lipid synthesis and composition in microalgae, DD-induced stress may have a similar effect on lipids in *P. tricornutum.*

In addition to altering lipid composition, stress has been shown to elicit the production of polyunsaturated aldehydes in algae (Goulitquer et al., 2009). The most commonly-produced PUAs among microalgae are 2*E*,4*E/Z*-decadienal and 2*E*, 4*E/Z*,7*/Z*-decatriental (Miralto, 1999). Although *P. tricornutum* has not been shown to produce these aldehydes, it does produce similar inhibitory compounds (Pohnert et al., 2002). Given that stress has been shown to stimulate PUA production in other algae, treatment with DD may lead to the production of similar inhibitory compounds in *P. tricornutum.*

Finally, stress has been shown to affect gene expression in microalgae. Allen et al. (2008) found that iron starvation led to changes in expression for a variety of genes in *P. tricornutum*. These included genes involved in photosynthesis, nitrogen metabolism and response to oxidative stress. De Martino et al. (2011) found that low-temperature and hypo-saline stress led to differential expression of genes involved in stress responses and lipid metabolism in *P. tricornutum.* In addition, Mus et al. (2013) demonstrated that stress has an effect on gene expression in *P. tricornutum*. This study focused on gene expression as it relates to lipid accumulation in this species, and the authors found that transcripts for many enzymes that alter lipid composition were up-regulated under nitrate deprivation and pH stress. Since these abiotic stressors were shown to alter gene

expression in this diatom, DD stress may have a similar effect on gene expression, especially in relation to lipid biosynthesis and modification.

Chapter 2: Review of the Literature

EVOLUTION AND CHARACTERISTICS OF DIATOMS

Diatoms are an incredibly diverse group of phytoplankton that play a crucial role in the global carbon cycle and serve as a base for marine food webs (Armbrust, 2009; Smetacek, 1999). They range in size from a few micrometers to a few millimeters, and are often covered by a cell wall made of silica called a frustule. These organisms can exist as single cells or as chains of connected cells that, for the most part, reproduce asexually (for review, see Armbrust, 2009).

Diatoms are a member of the Stramenopiles, a group that includes diatoms, brown macroalgae and plant parasites. Stramenopiles are believed to have originated from a secondary endosymbiosis event, in which a eukaryotic heterotroph engulfed a red alga. The algal mitochondrion and nucleus were lost, and many algal nuclear and plastid genes were transferred to the host's nucleus (McFadden, 2001; Armbrust et al., 2004; Bowler et al., 2008). The sequencing of two diatom genomes further supported this idea of secondary endosymbiosis and revealed a great deal about their evolutionary history. The first diatom genome to be sequenced was that of *Thalassiosira pseudonana,* a diatom that is considered widespread, residing in freshwater, brackish, and coastal habitats (Armbrust et al., 2004; Kipp, McCarthy, and Fusaro, 2013). *T. pseudonana* from Moriches Bay (Long Island, New York) was used for gene sequencing by Armbrust et al. (2004). Researchers found that about half of *T. pseusdonana* proteins had similarity to homologs in plant, red algal, and animal genomes. However, the other half could not be assigned functions because they did not bear similarity to genes from other organisms. Therefore, the authors explain, diatoms have several diatom-specific characteristics that most likely contribute to their ability to survive in a variety of sub-optimal conditions.

The second diatom genome to be sequenced was that of *Phaeodactylum tricornutum*. This diatom is found in many areas of the world, usually in coastal areas with large fluctuations in salinity (JGI, 2013). Bowler et al. (2008) discovered that *P. tricornutum* shares 57% percent of its genes with *T. pseudonana*, many of which are not present in other sequenced eukaryotes. The divergence in the genes of these two species shows that diatoms have been evolving at a rapid rate as compared to other groups of organisms. Another interesting finding was that many of the genes found in the *P. tricornutum* genome are the result of gene transfers between bacteria and diatoms. According to the authors, these genes are probably responsible for many of the unique metabolic capabilities of diatoms.

The "mix-and-match" nature of diatom genes has made it possible for these photoautotrophs to utilize nutrients in novel ways. In addition, unusual combinations of genes have provided diatoms with the ability to sense external stimuli in aquatic environments (Scala et al., 2002; Bowler et al., 2008). Clearly, the complex evolutionary history of diatoms has equipped them with a wide array of characteristics that enable these organisms to survive in a number of diverse environments. This unique combination of characteristics, according to Bowler et al. (2008), may explain why diatoms have come to dominate marine ecosystems in such a brief period of time.

PHAEODACTYLUM TRICORNUTUM AS A MODEL SPECIES

The sequencing of the *Phaeodactylum tricornutum* genome, along with the generation of over 130,000 expressed sequenced tags from *P. tricornutum* cells grown in several different conditions, established this species as a model species for the study of diatom biology (Scala et al., 2002; Bowler et al., 2008; Maheswari et al., 2010). In addition to these resources, a variety of molecular tools for the study and transformation

of this species have been developed. Transformation vectors for *P. tricornutum* were designed by Zaslavskaia et al. in 2000. In 2007, Siaut et al. identified several reference genes for use in gene expression studies of *P. tricornutum*. In addition, this group described expression vectors that could be used for high-throughput tagging and overexpression studies. De Riso et al. (2009) developed techniques for gene silencing in *P. tricornutum*, and reported the first phenotypic characterization of a diatom mutant. These tools, along with the ease with which *P. tricornutum* can be cultured, make it the species of choice for many researchers interested in diatom biology.

Other notable characteristics of *P. tricornutum* are its ability to exist as four possible morphotypes and its poorly silicified cell wall (De Martino et al., 2011; Lewin and Philpott, 1958). De Martino et al. (2011) found that stress can cause *P. tricornutum* cells to transform from one morphotype to another, and that this conversion is accompanied by changes in gene expression. When conditions are suboptimal, triradiate and fusiform cells convert into oval or round cells that secrete mucilage and aggregate into structures called biofilms. The abundance of a given morphotype, therefore, can serve as an indicator of stress in *P. tricornutum*, and may provide insight to how this species responds to environmental conditions.

LIPID BIOSYNTHESIS AND COMPOSITION

Because microalgae produce many valuable lipids, the process of lipid biosynthesis in these organisms is currently the subject of much investigation (Harwood and Guschina, 2009). Fatty acids are lipids that are important for membrane structure and energy storage (Ryall, Harper, and Keeling, 2003). Fatty acid biosynthesis has been well-characterized in plants, and animals, and yeast (Harwood, 1995; Pereira, Leonard, and Mukerji 2003; Wakil, Stoops, and Joshi, 1983). Fatty acid biosynthesis in microalgae

bears some similarities to pathways in plants and animals, but some aspects of this process are unique to this group (Domergue et al., 2003; Guschina and Harwood, 2006).

Fatty acid biosynthesis begins with conversion of acetyl CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) (Alberts and Vagelos, 1972). Malonyl-CoA is then transferred to an acyl carrier protein, forming malonyl-ACP. Malonyl-ACP is condensed with more acetyl-CoA, reduced, dehydrated, and reduced again to form an acyl-ACP that is four carbons in length. The carbon chain is extended by two carbons at a time by condensing another malonyl-ACP with the acyl-ACP and repeating the four-step reaction cycle (Ryall et al., 2003). The enzymes responsible for these reactions are collectively referred to as the fatty acid synthase (FAS). In animals and fungi, fatty acids are synthesized by one large multifunctional protein in the cytosol called a Type I FAS. In bacteria and plants, on the other hand, fatty acids are synthesized by a Type II FAS made up of separate enzymes (Harwood, 1996). Like plants, algae achieve fatty acid biosynthesis through a Type II FAS localized in the chloroplast (Ryall et al., 2003). In *P. tricornutum*, this multi-enzyme complex is believed to be responsible for production of saturated fatty acids with lengths of 14, 16, and 18 carbons (Domergue et al., 2003). These fatty acids are referred to as 14:0, 16:0, and 18:0, with the first number representing the number of carbons present, and the second number representing the number of double bonds in the carbon chain.

The fatty acids produced by ACC and the fatty acid synthase can be used directly in the chloroplast to produce structural lipids of photosynthetic membranes, or be exported to the cytosol for use in membrane lipid or TAG production (Ohlrogge and Browse 1995). In *P. tricornutum*, the major membrane lipids are the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulphoquinovosyldiacylglycerol (SQDG), and the phospholipids phosphatidylglycerol

(PG), and phosphatidylcholine (PC). These membrane lipids contain various combinations of medium- and long-chain fatty acids, especially C-16 and C-20 fatty acids (Arao, Kawaguchi, and Yamada, 1987).

In many species of plants and algae, high levels of mono- and polyunsaturated fatty acids accumulate in membrane lipids. In plants, a series of membrane-bound desaturases in the chloroplast and ER are responsible for converting 16:0 and 18:1 acyl groups to the major PUFAs $16:3^{\Delta 7,10,13}$ and $18:3^{\Delta 9,12,15}$. The pathway responsible for the production of $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ is located in the ER and is called the "eukaryotic pathway". The pathway that produces $16:3^{\Delta 7,10,13}$ is termed the "prokaryotic pathway", and occurs in the chloroplast. Each pathway utilizes a distinct set of fatty acid desaturases that differ in lipid substrates and electron donors (Ohlrogge and Browse, 1995; Domergue et al., 2003).

The synthesis of polyunsaturated fatty acids in diatoms is less well-understood than that of plants or animals, but the cloning and characterization of several fatty acid desaturases from *P. tricornutum* by Domergue et al., (2002; 2003) was an important step in elucidating lipid biosynthesis in diatoms. According to Domergue et al. (2003), the major fatty acids in *P. tricornutum* are 16:0, $16:1^{\Delta 9}$, $16:3^{\Delta 6,9,12}$ and $20:5^{\Delta 5,8,11,13,17}$. The high proportion of 20:5, or eicosapentaenoic acid (EPA), in *P. tricornutum* is one of the reasons researchers have focused their attention on this species (Domergue et al., 2002). EPA has been shown to have a variety of beneficial properties. This PUFA can prevent insulin resistance, has anti-inflammatory effects, and induces cell apoptosis in some types of cancer cells (for review, see Siriwardhana et al., 2012). In addition, Desbois et al. (2008) showed that EPA from *P. tricornutum* has antibacterial effects on a variety of pathogenic bacteria, including multidrug-resistant *Staphylococus aureus* (MRSA).

Domergue et al. (2002; 2003) cloned and characterized four fatty acid desaturases found in *P. tricornutum*. In these studies, yeast expressing desaturase genes were supplied with exogenous fatty acids to determine the specificity of each desaturase. DNA sequences for two of the desaturases, PtFAD6 and PtFAD2, were fused with the EGFP gene to determine the cellular location of these enzymes. EGFP fluorescence was colocalized with chlorophyll fluorescence for PtFAD6, and PtFAD2 appeared to be located in the cytoplasm (Domergue et al., 2003). Figure 1 shows the hypothetical compartmentalization of fatty acid desaturation in *P. tricornutum.* The "prokaryotic pathway", located in the chloroplast, is believed to be responsible for the production of 16:3 fatty acids, and the "eukaryotic pathway", located in the ER, is the believed to be responsible for EPA production. In contrast to plants, which primarily contain 18:1, 18:2, and 18:3 fatty acids, 18-carbon fatty acids have only been found in small amounts in *P. tricornutum*. This may indicate, according to Domergue et al. (2003), that these diatoms have developed very efficient mechanisms for converting these fatty acids into EPA.

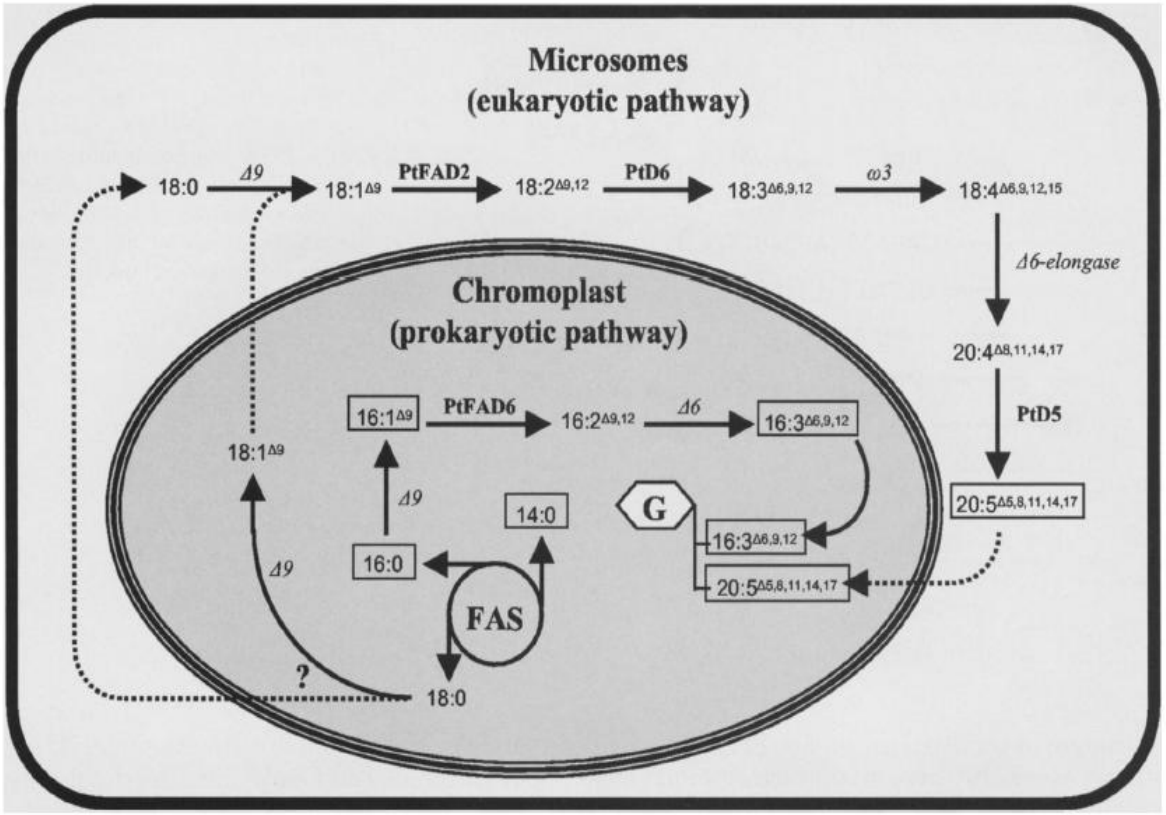


Figure 8. Hypothetical compartmentalization of fatty acid metabolism in *P. tricornutum* cells. The main fatty acids present in *P. tricornutum* (see Fig. 1) are framed. Desaturase genes that have been cloned are marked in bold, whereas putative desaturase (Δ6, ω3, and Δ9) and elongase are indicated with italics. Fatty acid modifications are indicated by arrows, and possible exchange reactions are indicated by dashed lines. The backbone with the framed G refers to the major glycolipids found in the plastid of *P. tricornutum* (monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and suphoquinovosyldiacylglycerol). Thioester- or lipid-linked desaturation is not differentiated and actually not known in detail. FAS, Fatty acid synthase.

Figure 1: Hypothetical compartmentalization of fatty acid desaturation in *P. tricornutum* (Domergue et al., 2003)

Under normal conditions, fatty acids are produced mainly for esterification into membrane lipids. However, in stressful or unfavorable conditions, lipid synthesis may be shifted away from membrane lipid synthesis and toward the formation of neutral lipids called triacylglycerols, or TAGs. These lipid bodies are synthesized in the ER, and serve as a form of carbon and energy storage (Hu et al., 2008). A good deal of research has

focused on increasing the production of TAGs in microalgae, because these lipids can be easily converted to biodiesel through transesterification (Chisti, 2007).

EFFECTS OF STRESS ON LIPID COMPOSITION

The effect of stress on lipid composition in microalgae has been well-documented, especially as it pertains to TAG production (Hu et al., 2008; Sharma et al., 2012). The lipid content and composition of microalgae is often altered when these organisms are exposed to environmental stressors such changes in nutrient supply, temperature, pH, or light (for review, see Sharma et al., 2012). In general, stress tends to lead to increases in overall lipid composition. However, large-scale comparisons of lipid production in many species show that these changes are specific to the type of stress and species of algae being considered (Hu et al., 2008; Sharma et al., 2012). Therefore, it is important not to over-simplify the effects of these stressors on lipid composition and production.

Nitrogen starvation is one stressor that has been shown to have a significant effect on lipid composition in microalgae (Alonso et al., 2000; Hu et al., 2008). Alonso et al. (2000) found that nitrogen starvation caused saturated and monounsaturated fatty acids such as 16:0 and 16:1 to accumulate in *P. tricornutum*. When nitrogen was decreased to 1/5 of the standard concentration, 16:0 fatty acids increased from 10 to 27% of biomass, and 16:1 fatty acids increased from 23 to 30%. At the same time, levels of polyunsaturated fatty acids, such as 16:3 and 20:5 decreased. 16:3 fatty acids decreased from 10 to 2%, and 20:5 decreased from 25 to 15%. In addition to changes in fatty acid composition, Alonso et al. (2000) also found that nitrogen deprivation caused major changes in lipid class composition. Nitrogen-deprived cultures had lowered levels of galactolipids (21 to 12%) and increased amounts of phospholipids and neutral lipids (6 to

8% and 73 to 79%, respectively). TAGs showed the greatest increase under nitrogen starvation, increasing from 69 to 75%. These findings imply that *P. tricornutum* responds to changes in its environment by restructuring its lipid classes. However, the authors point out, these changes are greatly dependent on the age of the culture, and distinguishing between the effects of stress and culture age may be difficult.

Temperature has also been shown to affect fatty acids in diatoms. Decreasing temperature leads to increases in PUFAs in *P. tricornutum* and other microalgae (Jiang and Gao, 2004). This is not surprising, given that many bacteria and plants exhibit the same response to cold temperatures (Harris and James, 1968; Marr and Ingraham, 1962). In a study of developing soybean seeds and temperature alteration, Cheesbrough (1989) found that desasturase enzymes were quickly modulated in response to low temperatures, but fatty acid synthesis enzymes were not.

In a study of the effects of nitrogen source and UV on *P. tricornutum* and *Chaetoceros muelleri*, Liang et al. (2005) discovered that UV radiation (UVR) caused changes the desaturation level of lipids in these diatoms. Cells exposed to UVR had higher levels of PUFAs and lower levels of SFAs than a control. Like many other studies, this study also confirmed that nitrogen was a major factor in fatty acid composition in diatoms, as nitrogen source had a greater effect on fatty acid composition than UVR.

Mus et al. (2013) found that nitrogen deprivation and elevated pH led to increases in lipid content in *P. tricornutum*. This group also analyzed gene expression levels under these conditions for a variety of gene transcripts. They found that the expression of genes for key enzymes in fatty acid synthesis was either unchanged or down-regulated under stress. However, phospholipase genes, as well as genes coding enzymes involved lipid metabolism and TAG production, were up-regulated, indicating that stress led to

increases in the expression of genes for enzymes that alter existing lipids, but not in expression of fatty acid synthesis genes.

In a review of high lipid induction in microalgae, Sharma et al. (2012) summarized the effects of several stressors on lipids in a variety of microalgal species. From this review, it is apparent that UV exposure and low temperature stress bring about increases in PUFAs in many microalgae. Conversely, phosphorous limitation, pH stress, and salinity stress lead to increases in saturated and monounsaturated fatty acids in many of the species studied. All the sources of stress discussed in this review were shown to increase lipid content in one or more species of microalgae, and many of them brought about increases in levels of TAGs.

This trend toward TAG accumulation under stress has been documented in many microalgal species, and several reasons for this trend have been proposed. TAG lipid bodies serve as a storage body for carbon and energy, and sequester excess electrons that have built up in the electron transport (ETC) chain as a result of reduced growth. Under stressful conditions, excess electrons accumulate in the photosynthetic electron transport chain, and over-production of reactive oxygen species can cause damage to membrane lipids and other molecules. TAG synthesis consumes about 24 NADPH from the electron transport chain, which alleviates stress on the over-reduced ETC. Additionally, TAG synthesis may also help detoxify membrane lipids by utilizing toxic fatty acids excluded from membrane lipids (Hu et al., 2008).

LIPID METABOLISM AND OXYLIPIN PRODUCTION

Diatoms have long been known to have a deleterious effect on the reproduction of copepods that graze on them (Miralto et al., 1999). This effect has been linked to a group of secondary metabolites called oxylipins that are produced upon wounding (Pohnert et

al., 2002). When cells are crushed or damaged, fatty acids are released from membrane lipids by the action of enzymes called lipases. Lipases are most likely compartmentalized from membrane lipids in order to prevent self-toxicity and provide "on-demand" response to wounding from predators (Pohnert, 2002; Pohnert et al., 2002). In the diatom *Thalassiosira rotula*, the enzyme phospholipase A_2 was found to be responsible for the release of fatty acids in wounded cells. This enzyme releases C-20 fatty acids from phospholipids when the cell membrane is compromised (Pohnert, 2002). However, other enzymes may also be involved in cleaving fatty acids from membrane lipids as well. Cutignano et al. (2006) reported that glycolipids, which are found in the chloroplast, are the main source of fatty acids for oxylipin production in *T. rotula*. Therefore, glycolipases, as well as phospholipases, must be involved in oxylipin production in diatoms.

The next step in oxylipin production is the conversion of free fatty acids to hydroperoxide products by the action of iron-containing enzymes called lipoxygenases (Pohnert et al., 2002). Lipoxygenase, or LOX, pathways have been well-described in higher plants and animals (for review, see Brash, 2013). In plants, lipoxygenases are a part of the jasmonate pathway. Jasmonates are a group of secondary metabolites that originate from membrane lipids (Creelman and Mullet, 1997). In plants, wounding and stress result in the release of α-linoleic acid (LA) by phospholipases, and LOX enzymes convert LA to hydroperoxy acids that are the precursors to a variety of metabolites. These metabolites, known as jasmonates, play a role in defense against diseases and predators, as well as in growth and development (Turner, Ellis, and Devoto, 2002; Wasternack, 2007). LOX has also been implicated in important pathways in humans. For example, the action of 5-LOX enzymes in leukocytes leads to production of leukotrienes, which cause airway constriction and inflammation (Drazen, Israel, and O'Byrne, 1999).

The final steps in the oxylipin synthesis pathway are mediated by hydroperoxide lyases, which cleave hydroperoxy acids to produce aldehydes and oxoacids (Pohnert et al., 2002). In diatoms, the main products of this phospholipase/lipoxygenase/lyase pathway are the polyunsaturated aldehydes (PUAs) 2*E*,4*E/Z*-decadienal (decadienal, or DD) and 2*E*, 4*E/Z*,*7/Z*-decatriental (decatrienal) (Miralto, 1999). *P. tricorntum* has not been shown to produce these specific PUAs, but the oxylipins 9-oxo-(5*Z*,7*E*)-nonadienoic acid (9-ONDE) and 12-oxo-(5*Z*,8*Z*,10*E*)-dodecatrienoic acid (12-ODTE) have been isolated from this species (Pohnert et al., 2002). These aldehydic acids share a common structural element with other oxylipins – a Michael acceptor. This structural element consists of a α,β,γ,Δ-unsaturated aldehyde group. Molecules with this structure are unstable, and can form adducts with nucleophiles (Pohnert et al., 2002). This structure is believed to be responsible for the effect that diatom diets have on copepod reproduction, such as disrupted mitosis during embryogenesis, and the induction of programmed cell death in embryos (Ianora et al., 2004; Miralto et al., 1999).

ROLES OF PUAS

Recently, it was discovered that PUAs are produced by intact diatoms and other algae (Goulitquer et al., 2007; Ribalet et al., 2007a). These findings suggest that PUAs such as decadienal and decatrienal may function in signaling pathways as well as in defense. Ribalet et al. (2007a) found that PUA production was enhanced in nutrient-stressed and aging cultures of the diatom *Skeletonema marinoi*. The authors of this study explain that PUAs may be produced by diatoms during bloom events when nutrients are depleted. This stress signal could then be propagated through the population, triggering cell death and terminating the bloom.

Goulitquer et al. (2007) obtained similar results when the brown algal kelp *Laminaria digitata* was exposed to both biotic and abiotic stress. PUA production increased in response to abiotic stress such as excess copper, and biotic stress, mimicked in this study by exposure to oligogluronate, which induces an oxidative burst in cells. This is an important study, because it confirms the fact that biotic stressors, as well as abiotic stressors, influence PUA production in algae.

Other important studies that furthered understanding of the roles of PUAs were those of Vardi et al. (2006; 2008). In 2006, Vardi et al. revealed that DD exposure induced nitric oxide (NO) production in the diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum*. In diatoms, as in other species, high levels of NO can initiate programmed cell death (Delladonne, 2005; Vardi et al., 2006). However, Vardi et al. (2006) also found that cells exposed to sub-lethal levels of DD developed resistance to future DD exposure. These pre-treated cells experienced lower levels of cell death than non-pretreated cells when exposed to lethal levels of DD. This led Vardi et al. (2006) to postulate that DD operates as an "infochemical" in marine ecosystems, providing a stress-surveillance system during algal blooms.

In 2008, Vardi et al. identified a gene in *P. tricornutum* that regulates the production of nitric oxide in response to stress. Overexpression of the *PtNOA* gene in transgenic clones led to enhanced NO production when these cells were exposed to decadienal. Overexpressing lines also experienced impaired growth and reduced surface adhesion as compared to wild-type cells. Therefore, the authors argue, *PtNOA* modulates sensitivity to stress-signaling molecules. This idea was further supported by the finding that superoxide dismutase proteins, which manage reactive oxygen species, were decreased in the transgenic lines. This indicates a reduced capacity for coping with stress. In addition, metacaspases, which play role in programmed cell death, were up-regulated

in these cells. Together, these findings support the hypothesis that diatoms detect aldehydes by means of a NO-based system, and that this system is involved in stress and death pathways (Vardi et al., 2008).

Because diatom-derived PUAs have a negative effect on diatoms and a range of other aquatic organisms, an allelopathic role has also been suggested for these molecules (Ribalet et al., 2007b; Leflaive and Ten-Hage, 2009). Characteristics such as cell size, membrane permeability, and cell wall structure may make some species more susceptible to the effects of PUAs than others. For example, phytoplankton that are relatively small in size and those lacking cell walls were shown to be more sensitive to PUA exposure than others (Ribet et al., 2007b). Not surprisingly, Ribalet et al. (2007b) also found that diatoms are less sensitive to the PUAs that they themselves produce than PUAs produced by other diatoms. This species-specific response to PUAs suggests that these aldehydes may shape species interactions and competition in aquatic ecosystems.

EXPERIMENTAL RATIONALE

The effects of abiotic stressors on lipid composition have been thoroughly studied in microalgae (Sharma et al., 2012). In addition, stress has been shown to have an effect on lipid-related gene expression in *P. tricornutum* (Mus et al., 2013). However, the effect of biotic stress on lipid composition and lipid-related gene expression in microalgae has not been extensively studied. Decadienal has been shown to function as a stress signal, and its effects on copepods, diatoms, and other aquatic species have been well-documented (Leflaive and Ten-Hage, 2009; Vardi et al., 2006). However the effect of this oxylipin on lipid-related gene expression in diatoms, to this author's knowledge, has not been studied. Therefore, the purpose of this study was to investigate the effect on decadienal-induced stress on gene expression in *P. tricornutum*. Because stress leads to

changes in lipid composition and gene expression in diatoms, it was hypothesized that DD exposure would lead to differential expression of genes involved in lipid biosynthesis in *P. tricornutum*. In addition, stress has been shown to promote the production of oxylipins by many diatoms, so genes coding for enzymes in the oxylipin pathway were also examined.

Chapter 3: Methods

Gene Selection Process

Genes were selected based on their putative involvement in lipid biosynthesis, desaturation, or oxylipin production (see below for rationales). For genes that have been previously described or characterized, nucleotide and protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) nucleotide and protein databases (http://www.ncbi.nlm.nih.gov/). For genes that have been proposed but not characterized, searches of the The *Phaeodactylum* Digital Gene Expression Database (http://www.diatomics.biologie.ens.fr/EST/) were performed, and sequences from other organisms were compared to those of *P. tricornutum* using the NCBI Basic Logical Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Six genes were selected for investigation. These include two genes that encode enzymes involved in *de novo* fatty acid synthesis, one that codes for an ER-localized desaturase, one that putatively codes for a plastid-localized desaturase, and two that are believed to be involved in oxylipin production.

Genes Selection Rationales

What follows is a brief description of each gene product, and the rationale for its selection.

ACC1 (PHATRDRAFT_54926)

The product of this gene is a putative acetyl-coa carboxylase (ACC), which catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate with the hydrolysis of ATP to ADP + P. The ACC sequence used in this study was retrieved from the NCBI nucleotide database. The sources given by NCBI for this sequence are Bowler et al. (2008) and Grigoriev et al. (2008). A BLAST query shows that this sequence is

similar to an acetyl-CoA carboxylase gene from the green algae, *Ostreococcus tauri*, with an E-value of 1e-96 and 74% identity.

This gene was selected for this study because the conversion of acetyl-CoA to malonyl-CoA by ACC represents the first committed step of fatty acid biosynthesis (Alberts and Vagelos, 1972). In addition, overproduction of this carboxylase has been linked to increases in fatty acid synthesis (Davis, Solbiati, and Cronan, 2000). Therefore, analysis of this gene will reveal whether DD has an effect on *de novo* fatty acid synthesis in *P. tricornutum*.

FabI (PHATRDRAFT_10068)

FabI encodes enoyl-ACP reductase, a subunit of the fatty acid synthase II complex. This complex is made up of 6 enzymes that that together synthesize fatty acids in the plastid. (Ryall, Harper, and Keeling, 2003). The fatty acid synthase is believed to be responsible for the production of 14:0, 16:0, and 18:0 fatty acids in *P. tricornutum*. 16:0 fatty acids are one of the major fatty acids in *P. tricornutum*, making up 16.6% of total FAs. In addition, 16:1 and 16:3 fatty acids, which are derived from the desaturation of 16:0, are also among the most abundant in *P. tricornutum*, accounting for 25.9% and 10.4% of FAs, respectively. 18:0 is also an important fatty acid because it is the precursor to the 20:5 fatty acid eicosapentanoic acid (EPA) (Domergue et al., 2003). FabI, or enoyl-ACP reductase, catalyzes the final reduction in the fatty acid biosynthesis process. This gene has been shown to be a major point of regulation for bacterial and plastid fatty acid biosynthesis, and is a common drug target (Ryall, Harper, and Keeling, 2003). Ryall, et al. characterized the FabI gene sequence in the *P. tricornutum* through phylogenetic analysis that compared FabI proteins among several chromist species. The sequence identified by this group was retrieved from the NCBI database for use in this study. If DD

exposure results in changes in the activity of the type II FAS in *P. tricornutum*, the expression of this gene should be altered.

Palmitoyl-CoA Delta-9 Desaturase (PHATRDRAFT_22510)

This gene is thought to code for a delta-9 desaturase that converts 16:0, or palmitic acid, to $16:1^{\Delta 9}$, or palmitoleic acid. An mRNA sequence for a palmitoyl-CoA delta-9 desaturase submitted by Kim et al., (2005) for the *P. tricornutum* strain KMCC B-128 was found on the NCBI nucleotide database (accession number JX195611.1). A BLAST query revealed that this sequence is 100% identical to a sequence from the strain CCAP 1055/1, the strain used in this study. The references cited for this sequence are the same as those for ACC1. This gene was chosen for inclusion in this study because it is believed to be responsible for the desaturation of 16:0 lipids to produce 16:1 lipids, which are among the most prevalent in *P. tricornutum*. According to Domergue et al., (2003), it is likely that the desaturation of 16:0 fatty acids occurs in the plastid. Therefore, this gene represents plastid-localized desaturation of one of the major fatty acids in *P. tricornutum*. Like other types of stress, DD-induced stress may cause alterations in the desaturation of fatty acids (Alonso et al., 2000; Liang et al., 2005). If this is so, genes coding for desaturase enzymes may be up- or down-regulated after DD exposure.

PtD5a (PHATR_46830)

PtD5a encodes a delta-5 desaturase that has been functionally characterized in yeast by Domerge, et al. (2002). In this study, Domergue et al. demonstrated that this delta-5 desaturase is responsible for the conversion of $20:4^{\Delta 8,11,14,17}$ to $20:5^{\Delta 5,8,11,14,17}$, or Eicosapentanoic acid (EPA) in the endoplasmic reticulum. EPA is the most prevalent fatty acid in *P. tricornutum*, making up 30.2% of total fatty acids. This PUFA is also important in many ways, especially in human health and nutrition (Desbois et al., 2009;

Siriwardhana et al., 2012). It has also been shown to serve as a precursor for the production of oxylipins that function in competition and communication (Ribalet et al., 2007b; Vardi et al., 2006). This gene represents a major desaturation step in the endoplasmic reticulum, and will therefore help reveal whether DD exposure has an effect on ER-localized fatty acid desaturation (Domergue et al. 2002). The references provided for this gene on the NCBI database are the same as those provided for ACC1.

Patatin-like phospholipase (PHATRDRAFT_46193)

The phospholipase sequence used in this study was found by performing a keyword search of the The *Phaeodactylum* Digital Gene Expression Database for the word "phospholipase". A protein from *Pan troglodytes* (chimpanzee) with the description "similar to intracellular membrane-associated calcium-independent phospholipase A2 gamma" appeared with an E-value of 2e-20 and the accession number XP_527859.1. When an NCBI BLAST search was performed comparing this protein to those from *P. tricornutum*, a protein with the accession number XP_002180454 was shown to have an E-value of 2e-40. This protein contains a region labeled "patatin-like phospholipase domain containing protein 8" (Pat_PNPLA8). Patatin is a storage protein found in potatoes, and Pat_PNPLA8 is part of the Patain_and_cPLA2 Superfamily. PNPLA8 is a Ca-independent myocardial phospholipase that catalyzes both phospholipase A1 and A2 reactions in humans (Marchler-Bauer et al., 2013). This phospholipase protein from *P. tricornutum* bears similarity to many other phospholipase proteins according to an NCBI BLAST query, especially that of *Thalassiosira pseudonana* CCMP1335 (accession number XP_002292259, E-value 1e-169).

This gene was chosen for examination because stress has been shown to prompt oxylipin production in diatoms, and the first step in this process is the release of free fatty

acids from membrane lipids (Goulitquer et al., 2007; Pohnert, 2002; Ribalet et al., 2007a). Phospholipase A_2 was found to be responsible for the release of C20 fatty acids after wounding in *T. rotula* (Pohnert, 2002), and the product of this gene bears similarity to phospholipase A_2 proteins in many other organisms. Goulitquer et al. (2007) found that biotic stress caused an increase in PUA production in algal kelp, so DD may also elicit production of oxylipins in *P. tricornutum*. If this is the case, transcription of this gene may be enhanced after treatment with DD. In addition, stress has been linked to increased lipid metabolism and remodeling of lipids. Phospholipases are involved in this remodeling, so DD exposure may lead to the up-regulation of this gene (Mus et al. 2013).

Lipoxygenase (CQ768401.1)

The sequence for the lipoxygenase gene used in this study was obtained from a patent submitted by Feussner, Senger, and Goebel in 2004 titled "Cloning and Characterization of a Lipoxygenase from *Phaeodactylum tricornutum*." This sequence is 100% identical to a lipoxygenase gene from *Physcomitrella patens*, according to an NCBI BLAST query. Interestingly, a BLAST query comparing this sequence to the genome of *P. tricornutum* does not yield any results. However, the sequence was still included in the study because another lipoxygenase gene from *P. tricornutum* was not able to be found through BLAST queries or through searches of the *Phaeodactylum* Digital Gene Expression Database.

Lipoxygenase was included in this study of gene expression under DD stress because lipoxygenase plays a role in oxylipin production. Lipoxygenase catalyzes the dioxygenation of free polyunsaturated fatty acids to produce hydroperoxy fatty acids that are precursors to volatile compounds produced by diatoms (Pohnert and Boland, 2002).

As described above, DD production may elicit oxylipin production in *P. tricornutum*, and as a result, the expression of lipoxygenase genes may be increased.

In a personal communication with Dr. Ivo Feussner (July 22, 2013), it was later revealed this sequence is in fact from *P. patens*, which is a species of moss. Dr. Feussner explained that after this patent was filed, researchers discovered that the *P. tricornutum* library from which this sequence was isolated was contaminated with cDNA from *P. patens.*

PRIMER DESIGN

Primers were created using PerlPrimer v1.1.21 software (http://perlprimer.sourceforge.net/). The primer temperatures of melting (Tm) were set at 58-60°C, with a difference of 1°C. Primer length was 20-24 bases, and amplicon size was restricted to 50-150 bases. Only primers with G-C content between 30 and 80% were considered, and primers containing runs of more than 3 repeats of 4 runs were excluded. Primers with ΔG values more negative than -4 kcal/mol were not considered, and ΔG values for extensible primer-dimers were between 0 and -0.15 kcal/mol for the primers that were selected. When possible, primers that spanned the intron/exon boundary with an overlap of 7 bases were chosen. Primers were analyzed for self-annealing using the Hairpin function of the Integrated DNA Technologies OligoAnalyzer 3.1. Primers that formed hairpin structures with ΔG values lower than -4 kcal/mol were not selected. The BLAST function of PerlPrimer was used to verify that primers were only highly homologous to the gene of interest, and not to any other *P. tricornutum* genes. Table 1 shows the primers used for qRT-PCR analysis of gene expression in this study.

Gene	Gene abbreviation	Forward primer	Reverse primer
PHATRDRAFT_54926	ACC1	AAGTGAGACTCACCTTAACGG	AATGGTAGGCATCAAGATCGT
PHATRDRAFT_10068	FabI	GGATGGTTCCCTAATGACGA	ATGGTGTATCCGTCCAATCC
PHATRDRAFT_22510	PD9	CAAATTGAACACCATCTGTTTCC	ATTGCTCGCATTATATCGGAC
PHATR_46830	PtD5a	ACCAATGCTCCTATTCAACGA	AACCAGTATCCAGCCAAGAC
PHATRDRAFT_46193	PLA	TGCACGATAGGTGTAATTGG	TGCTCTTATAAATTGTTGTCAAGG
CQ768401.1	LOX	GACGACTGTTATTTGGATACCT	GTTGAAGTCTGCTTTGTCCT

Table 1: Primers used for qRT-PCR analysis of gene expression

GROWTH CONDITIONS

Culture samples of *Phaeodactylum tricornutum* CCMP 2561 were obtained from the Pravasoli-Guillard National Center for the Culture of Marine Phytoplankton. An existing culture that had reached lag growth stage was used to inoculate a 500-ml culture grown in f/2 media. This culture was expanded into 1500-ml cultures grown in 2800-ml flasks. Cultures were grown under fluorescent light with an intensity of 6.5 kilolux in a 12:12 light:dark photoperiod. The temperature of the growth chamber in which flasks were housed was 20°C. Cultures were swirled daily in order to keep cells in suspension. Fresh media was added approximately every 3-4 days when cell counts exceeded 1 x 10^6 cells/ml. Cell counts were obtained using a Reichert Bright-Line® hemacytometer.

DECADIENAL TREATMENT CONDITIONS

Cultures with densities of ~7 x 10^5 cells/ml were pooled in order to create a uniform culture. The pooled culture was then separated into nine 200-ml cultures in 500-ml flasks for the 2-hour treatment group. 2*E*,4*E/Z*-decadienal (Sigma-Aldrich) was dissolved in DMSO to create a 50mM DD stock. 80 µl of this stock was then added to

three replicate cultures to reach final DD concentrations of 20μM. This concentration was shown to be sub-lethal in a growth assay carried out by another member of the Mona Mehdy research group. This assay was part of a study that is currently in progress, but preliminary results from the work of Tanya Sabharwal showed that *P. tricornutum* cultures treated with DD at concentrations as high as 50 μM were able to recover and continue growing if fresh media was added after 6 hours of treatment. 80 μl of DMSO was added to three replicates to serve as a solvent control, and 80 μl of deionized water was added to three replicates that would serve as a water control. In order to allow time for centrifugation, one replicate from each treatment group was treated 40 minutes later than the first six replicates. Replicates in the 6-hour treatment group were prepared in a similar manner. Treatment of cultures was completed over the course of two days to allow time for RNA isolation. Therefore, the cell densities of the 2-hour and 6-hour treatment groups were slightly different. At the time of treatment, the 2-hour group had a cell density of 7.1×10^5 cells/ml, and the 6-hour group had a cell density of 7.8×10^5 cells/ml.

RNA ISOLATION

A protocol for RNA isolation was selected after attempting several preliminary RNA isolations with a variety of different methods. Initially, 500- and 250-ml cultures were centrifuged in a Beckman/Coulter Allegra x-22R high speed centrifuge at 10,000 rpm and 4°C. Pellets were then ground in liquid nitrogen using a mortar and pestle. Ground tissue was then added to 0.5 ml Trizol® Plant RNA Purification Reagent (Life Technologies) in 1.5-ml microcentrifuge tubes. RNA isolation was then carried out according to the manufacturer's instructions for small-scale RNA isolation. RNA samples obtained by this method were analyzed using a NanoDrop® 1000 Spectrophotometer and

found to have relatively low concentrations and purity. Gel electrophoresis was also used to evaluate the quality of RNA, and 28S and 18S bands could not be seen. Therefore, a new method for RNA isolation was attempted. In this method, pelleted cells were placed in Trizol® Plant RNA Purification Reagent in centrifuge tubes made to fit the QuiaCube. 0.5-mm zirconia/silica beads (Biospec Products) were added to the tissue/reagent mixture, and samples were oscillated in a Qiagen Tissue Lyser LT at 50 oscillations/second for 5 minutes. RNA isolation was then completed according to the Trizol® manufacturer's protocol, beginning with step 2. RNA samples that were extracted in this manner had higher concentrations and higher quality than those that were isolated by crushing in liquid nitrogen. Therefore, the bead-beater protocol was used for isolating RNA from the experimental groups.

GEL ELECTROPHORESIS

Denaturating formaldehyde/MOPS gels were prepared by boiling 0.5 grams of UltraPure™ Agarose from Invitrogen in 45 ml deionized water. Agarose solutions were then cooled to 50-60°C and 5 ml of NorthernMax® 10X Denaturating Gel Buffer was swirled into the solution. Gels were run in 1X Ambion NorthernMax MOPS Gel Running Buffer in a BioRad Mini-Sub® Cell GT under a fume hood. RNA samples were combined with equal volumes of 2X RNA loading dye from Thermo Scientific and heated to 70°C for 10 minutes. After chilling on ice, samples and were loaded into wells, and 75V of power was supplied until the tracking dye had traversed approximately half the distance of the gel. A Fermentas RibroRuler™ High Range RNA Ladder #SM1823 was loaded along with RNA samples in order to analyze RNA band size. Gels were imaged on a VisiDoc-It™ Imaging System from UVP.

cDNA Synthesis

cDNA was synthesized from total RNA using a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. For each RNA sample, 1 µg of RNA in 8 µl of RNAse-free water was DNAse-treated, then incubated at room temperature for 15 minutes. 1 µl of EDTA was added, and samples were heated to 65°C in a water bath for 10 minutes. A master mix containing 10X RT buffer, 25X dNTP Mix, 10X RT random primers, RNAse free water, and MultiScribe Reverse Transcriptase™ was added to the DNAse-treated RNA, then samples were placed in a BioRad iCycler™ 2x48 Well Reaction Thermal Cycler. The cycler was programmed with the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. cDNA was stored at -20°C until it was used in qRT-PCR reactions.

Primer Standardization

cDNA was prepared in amounts of 50, 10, 1, 0.1, 0.01, and 0.001 ng in 5 µl of deionized water for primer standardization. A no-RT sample (to which no reverse transcriptase was added) containing 50 ng in 5 µl of water was also synthesized in preparation for primer standardization. A mastermix containing deionized water, forward and reverse primers (10 µM), and *Power* SYBR® Green PCR Master Mix was prepared. 5 µl of each cDNA amount, as well as no-RT and water controls, were combined with 15 µl of mastermix, in triplicate, in a 384-well PCR plate. The plate was run in an Applied Biosystems ViiA™ Real-Time PCR system in the ICMB core facility at The University of Texas at Austin. The default thermal cycling conditions were selected, and a melt curve was added to the thermal profile. CT data was exported to an Excel spreadsheet, and graphs of average CT values versus log of cDNA amount were prepared. Melt curve analysis was used to confirm primer specificity.

REAL-TIME QRT-PCR ANALYSIS

For real-time qRT-PCR analysis, a mastermix containing deionized water, forward and reverse primers, and *Power* SYBR® Green PCR Master Mix was prepared. 15 µl of mastermix was combined with 5 µl of cDNA from each biological replicate, in triplicate, in a 384-well PCR plate. The plate was placed in an Applied Biosystems ViiA™ Real-Time PCR system in the ICMB core facility at The University of Texas at Austin, and run according to default thermal cycling conditions. Melt curve analysis was used to confirm primer specificity. CT data was exported to an Excel spreadsheet, and fold changes were calculated using the ΔΔCT method (Livak and Schmittgen, 2001). ΔCT values were calculated for each primer set by subtracting average CT values for a reference gene from average CT values for water control, solvent control, and DD-treated replicates. The reference gene used was that which codes for TATA-box binding protein, PHATR_10199. This gene has been suggested as a suitable reference gene by Siaut et al. (2007). ΔΔCT values were calculated by subtracting ΔCT values for water control replicates from ΔCT values for DMSO- and DD-treated replicates for each primer set. Fold changes were calculated using the formula $2^{(-\Delta\Delta CT)}$.

Data was only available for one DD-treated replicate from the 2-hour group, because RNA isolation had not been successful for two of the DD-treated replicates. Therefore, data from the 2-hour time point was not included in the graphs shown in the results section, because average fold changes could not be calculated for the DD-treated group, and standard error could not be shown for these data. RNA isolation was successful for at least two replicates from each treatment group at the 6-hour time point. Fold changes were calculated for two replicates from each treatment group, and bar graphs showing average fold changes for each experimental group were prepared. Standard error of the mean was calculated for each treatment group, and displayed as

standard error bars on each graph (Livak and Schmittgen, 2001). Two-tailed, two-sample unequal variance t-tests were used to determine p-values for fold changes in DD-treated groups as compared to solvent and water controls.

Chapter 4: Results and Analysis

RNA Sample Analysis

RNA samples were assessed for concentration and quality using a NanoDrop® spectrophotometer and by agarose/formaldehyde gel electrophoresis. RNA samples obtained using the liquid nitrogen procedure described in the methods section were shown to have RNA concentrations below 150 ng/ul, 260/280 ratios between 1.9 and 2, and 260/230 ratios below 1.25. Gel electrophoresis revealed that distinct 18S and 28S ribosomal RNA bands were not visible for these samples. Figure 2 shows a gel in which RNA isolated by the liquid nitrogen protocol and RNA isolated using the bead-beater protocol were simultaneously run. In lane 1, where samples isolated using the bead-beater technique were run, two bands at approximately 3-4 kb and 1.5-2 kb are visible, but in lanes 3 and 4, these bands are very faint if at all present. The appearance of a bright band with a low molecular weight in lanes 1 and 3 may suggest degradation of RNA. However, it was not clear what caused this degradation.

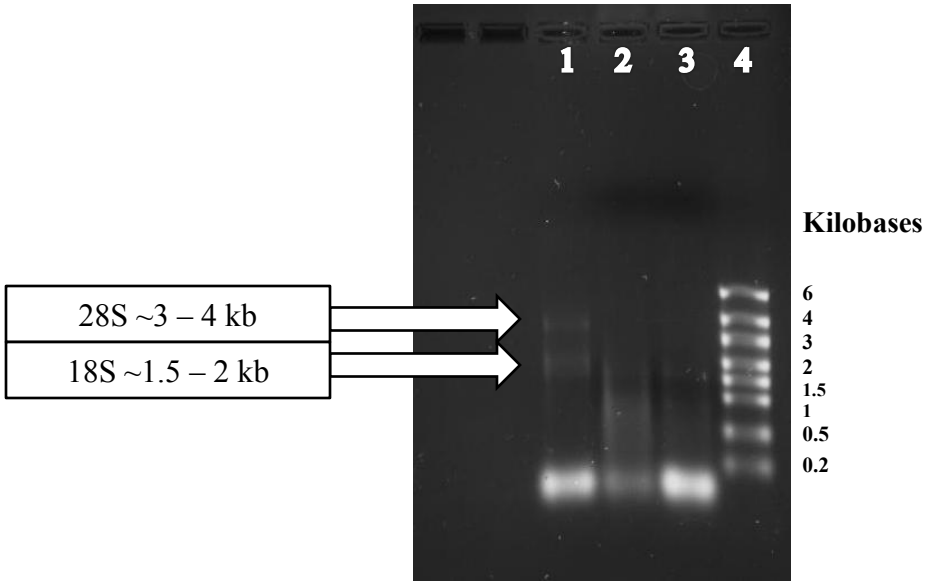


Figure 2: RNA isolated using bead-beater and liquid nitrogen techniques. Lane 1, bead-beater, lanes 2 and 3, liquid nitrogen.

RNA samples from the 2-hour experimental group were isolated using the bead-beater protocol described in the methods section. The purity of these samples was assessed on a NanoDrop® spectrophotometer, and three samples were found to have very low concentrations (11.9 – 82.6 ng/µl) and low 260/230 ratios (0.78 – 0.84). Therefore, these replicates, which included two replicates from the DD-treated group and one replicate from the control group, were not included in qPCR analysis. The remaining samples had concentrations ranging from 268.8 to 829.1 ng/µl, 260/280 ratios between 2.11 and 2.19, and 260/230 ratios from 1.17 to 1.80. The 260/230 ratios, which indicate carbohydrate contamination, were relatively low, so clean-up of these samples was considered. However, time did not allow for this, so these samples were used as-is for cDNA synthesis.

Figure 3 is an image of the agarose/formaldehyde gel on which the remaining replicates were run. 28S and 18S bands are visible for all samples.

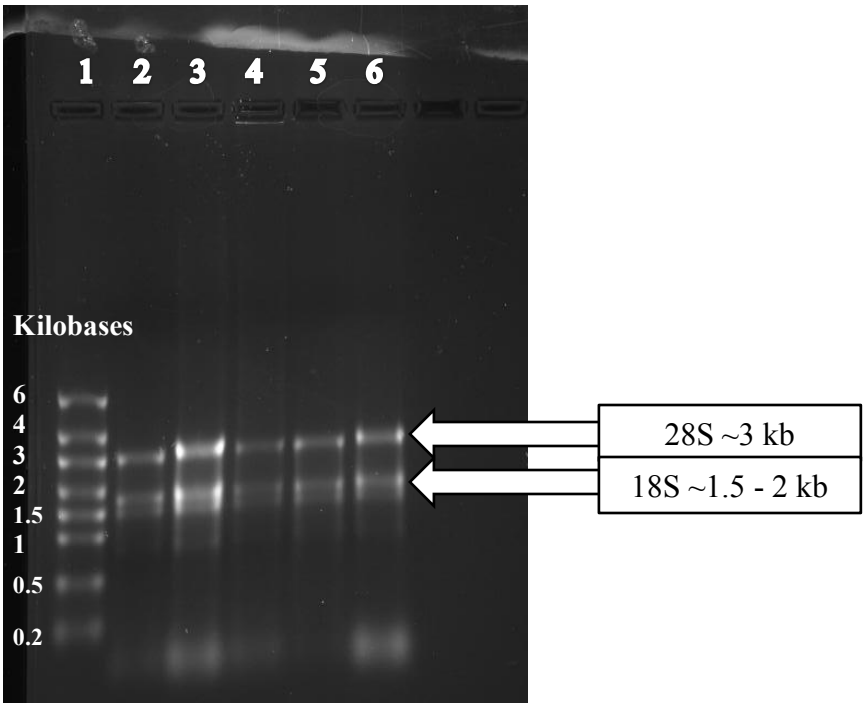


Figure 3: Gel electrophoresis of RNA samples from 2-hour group

Two replicates from the 6-hour experimental group also had low concentrations and purity, so these samples were excluded from qRT-PCR analysis. This included one replicate from the DD-treated group, and one replicate from the DMSO group. The 260/230 ratios for the remaining samples were higher than those from the 2-hour group, ranging from 1.57 to 1.94. 260/280 ratios for these samples were similar to those of the 2 hour group, varying between 1.99 and 2.19.

Gel electrophoresis of these samples showed similar results to samples from the 2-hour group, with visible 28S and 18S bands (see figure 4). Errors in loading RNA samples resulted in the faint bands seen in lanes 3 and 4.

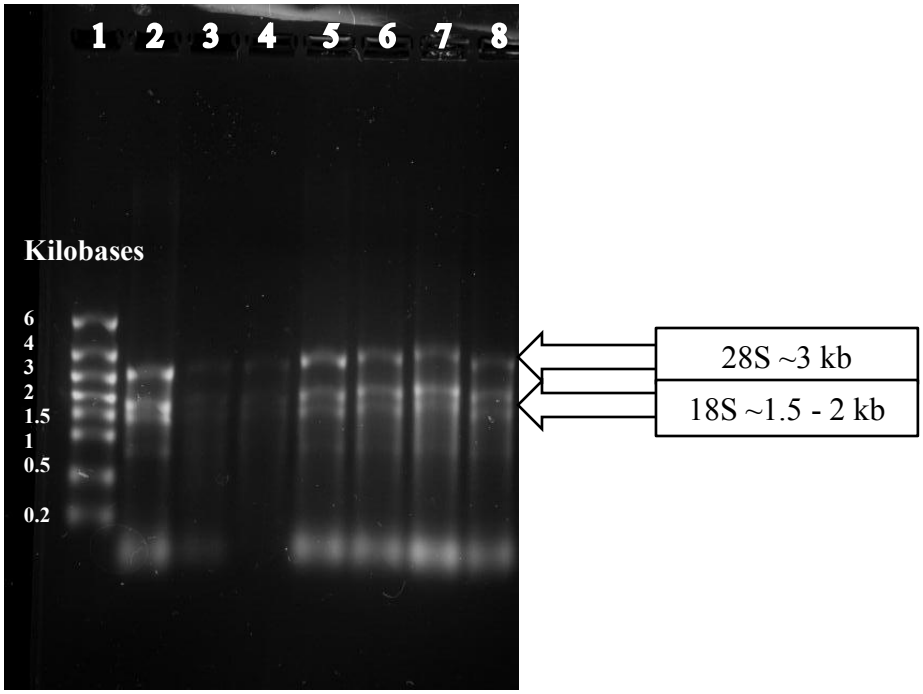


Figure 4: Gel electrophoresis of RNA samples from 6-hour group

PRIMER STANDARDIZATION

Graphs of primer standardization data were prepared by plotting the average CT value for each amount of cDNA template (Y axis) versus the log of template amount (X axis). In the case of FabI, palmitoyl-CoA delta-9 desaturase, and patatin-like phospholipase, average CT values for the template amount 0.001 ng are not shown, because more than one CT value was undetermined, so an average could not be calculated. All CT values for the LOX primer set were undetermined, indicating that these primers did not anneal to the cDNA template. Figure 5 displays the results for ACC1, FabI, Palmitoyl-CoA delta-9 desaturase (hereafter referred to as PD9), PtD5, and Patatin-like phospholipase (hereafter referred to as PLA) primer sets. Linear regression was performed, and R-squared values were calculated for each primer set. R-squared values were all above the minimum acceptable value of 0.99. In addition, the slopes of

linear regression lines were between -3.1 and -3.4 for all primers. Slope values between -3.58 and -3.10 indicate 90 – 100% efficiency. Therefore, all primer sets, except those for the LOX gene, were deemed acceptable for use in real-time qRT-PCR.

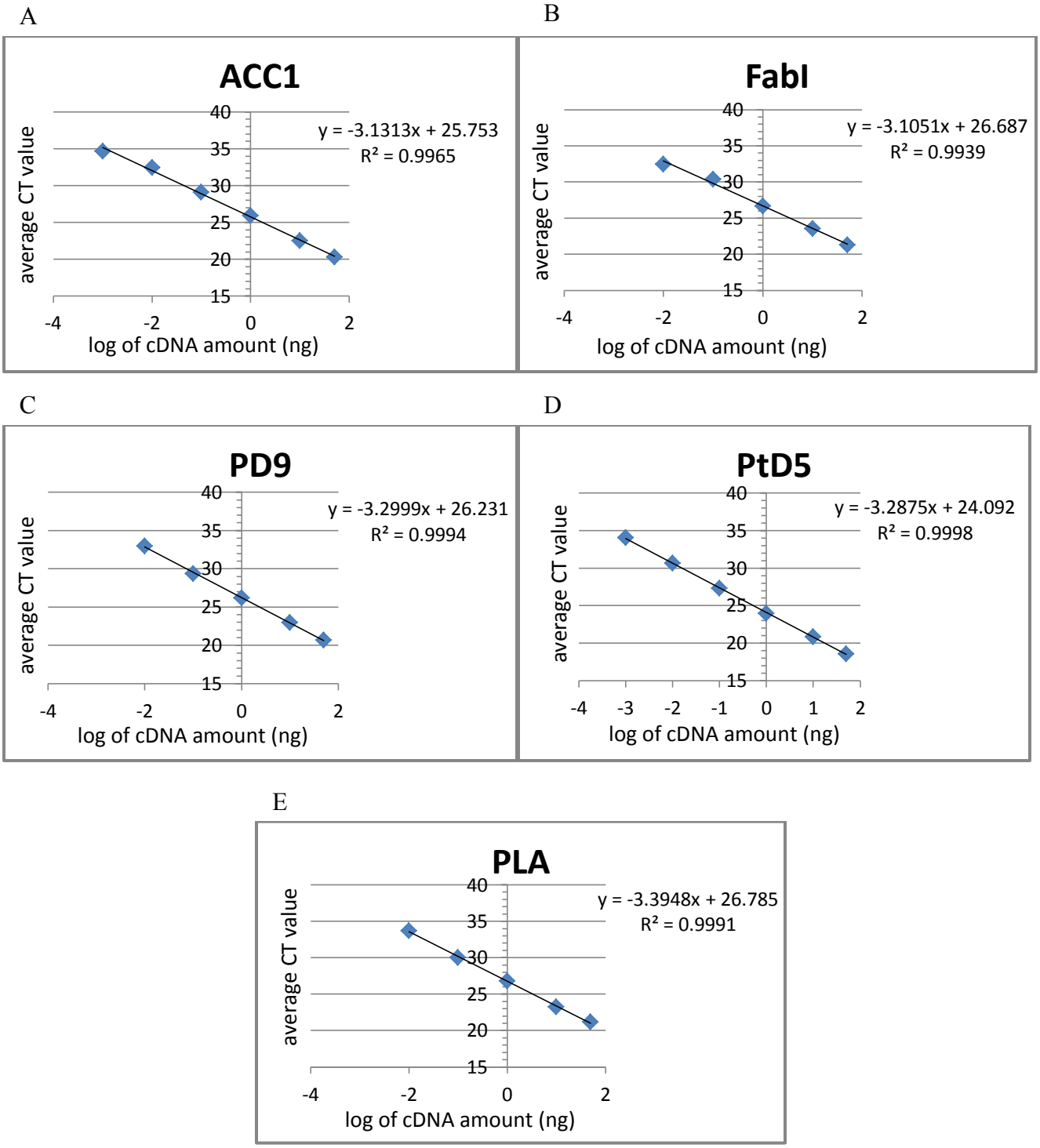


Figure 5: Standardization of primer sets. A, PHATRDRAFT_54926; B, PHATRDRAFT_10068; C, PHATRDRAFT_22510; D, PHATR_46830; E, PHATRDRAFT_46193; Average CT values are based on triplicate reactions.

REAL-TIME QRT-PCR ANALYSIS

Bar graphs showing average fold changes for two replicates from each treatment group at the 6-hour time point are shown in figure 6. Water control replicates were used as a control when calculating ΔΔCT values, so the fold change for this group is equal to 1. Fold changes greater than 1 indicate up-regulation of genes, and fold changes less than 1 indicate down-regulation. Standard error bars represent the standard error of the mean. Fold changes were only shown to be statistically significant for the PLA gene (p-value < 0.05).

The 6-hour DD-treated and solvent control groups showed down-regulation of the ACC1 gene. The fold change for the solvent control was 0.32 ± 0.11, and the fold change for the DD-treated group was 0.50 ± 0.28. Due to the relatively large standard error for these groups, it is not possible to conclude whether the gene was up- or down-regulated in DD-treated replicates as compared to the solvent control.

Expression of the FabI gene was increased in the solvent control group, which saw a fold change of 1.57 ± 0.33. The DD-treated group experienced a decrease in expression as compared to the water control, with a fold change of 0.61 ± 0.07. Therefore, the expression of the FabI gene was down-regulated in the DD-treated group as compared to both the water and the solvent control.

Expression of the PD9 gene was increased more than 12-fold (± 2.24) in the DD-treated cultures. The fold change of the solvent control group was 1.23 ± 0.20, so expression of the PD9 gene was up-regulated in the DD-treated group as compared to both water and solvent controls.

PtD5 gene expression was down-regulated as compared to the solvent control, but when the standard error is taken into account, the difference in expression is a modest one. The fold change in the DD-treated group as compared to the water control was 0.85

$\pm$ 0.27, and the fold change in the solvent control was 1.36 $\pm$ 0.17. Whether the expression of PtD5 was increased or decreased in the DD-treated group as compared to the water control is inconclusive.

As compared to the water control, expression of the PLA gene in the DD-treated group was decreased by a factor of more than 8. Expression in the solvent-control was similar to that of the water control, with a fold change of 0.94 $\pm$ 0.06. Therefore, PLA was down-regulated in comparison to both the solvent and water controls. This fold change was shown to be statistically significant (p-value < 0.05).

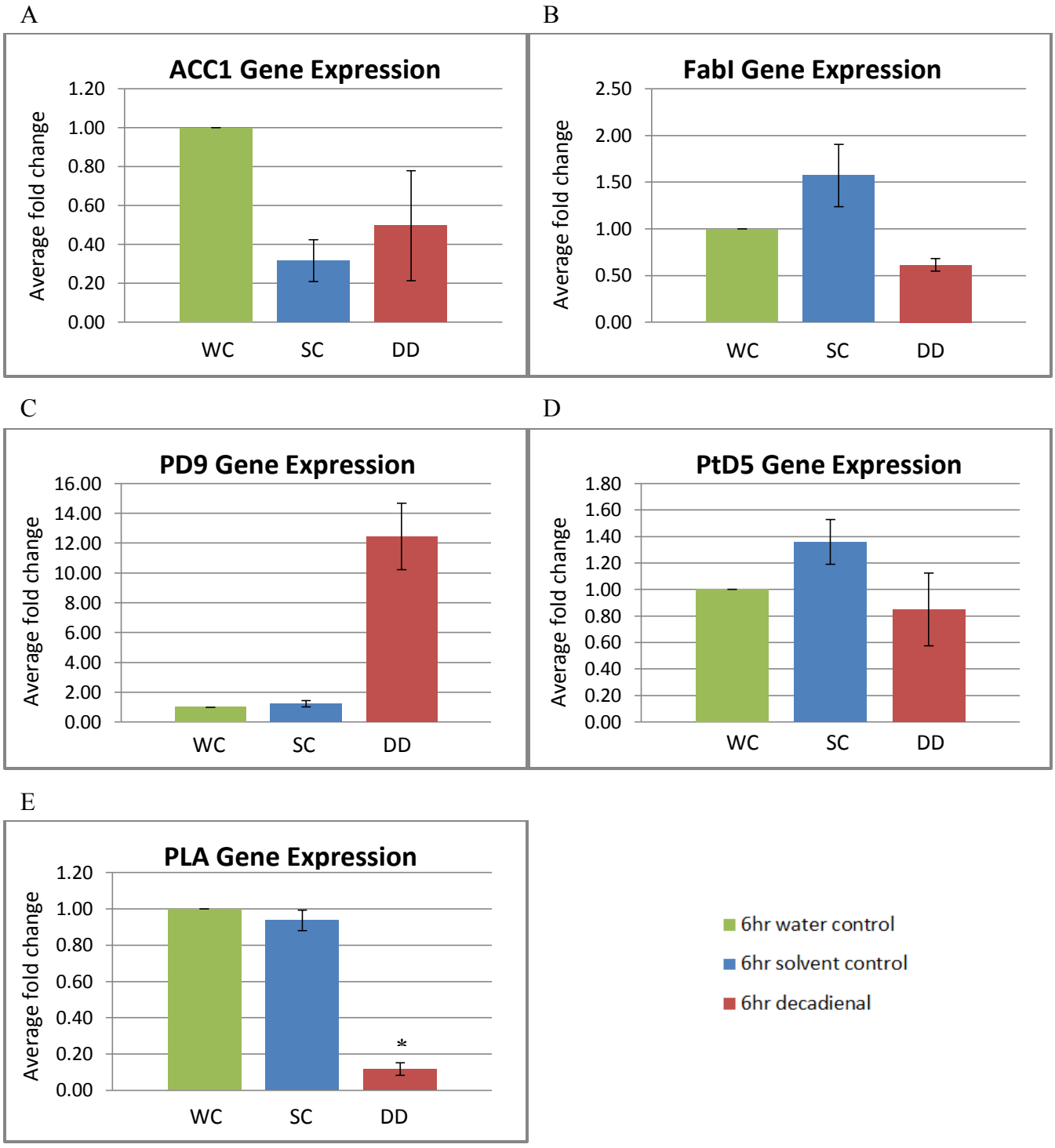


Figure 6: Average fold changes in gene expression. A, PHATRDRAFT_54926; B, PHATRDRAFT_10068; C, PHATRDRAFT_22510; D, PHATR_46830; E, PHATRDRAFT_46193. WC, water control; SC, solvent control; DD, decadienal-treated. *, $p < 0.05$ relative to water and solvent controls. n=2.

Chapter 5: Conclusions

The goal of this study was to gain insight into the effects of biotic stress on lipid-related gene expression in the model diatom, *P. tricornutum*. Many studies in this area have focused the effects of abiotic stressors, but little is known about the effects of biotic stress on gene expression related to lipid biosynthesis and metabolism. A deeper understanding of these pathways and the factors that influence them is necessary if microalgae are to be used as a feedstock for biofuel production.

Lipid Biosynthesis Genes

A conclusion about the expression of the ACC1 gene could not be made based on the data collected in this study. The expression of FabI was decreased as compared to the solvent control, but this change was not shown to be statistically significant. Future studies could correct for this by including a larger number of biological replicates. Decreased expression of the FabI gene is in agreement with previous research on gene expression under stress in *Phaeodactylum tricornutum*. Mus et al. (2013) found that expression of ACC1 and FabD, another subunit of the fatty acid synthase, was decreased or unchanged in *P. tricornutum* cells subjected to nitrate limitation and pH stress. This group discovered that genes coding for enzymes involved in the repurposing of lipids were up-regulated, but enzymes involved in fatty acid synthesis were not. In order to shed more light on the effects of stress on lipid biosynthesis, future studies should correlate changes in gene expression with changes in enzyme levels, and examine possible changes in the activity of these enzymes.

Lipid Desaturase Genes

The PD9 gene showed the greatest fold change, with a 12-fold increase in expression. Although this change was not statistically significant, this finding aligns with

previous research on the effect of some types of stress on fatty acid composition. For example, Alonso et al. (2000) found that the saturated and monounsaturated fatty acids 16:0 and 16:1 increased in nitrogen-deprived cultures. However, the opposite trend is true for other types of stress, such as UVR exposure and low-temperature stress. An increase in saturated and monounsaturated fatty acids may alter membrane lipids in a way that is beneficial in times of stress or herbivory. In addition, an increase in 16:1 fatty acids may aid in defense against infection, because 16:1 fatty acids from *P. tricornutum* have been found to have antibacterial properties (Desbois, Lebl, Yan, and Smith, 2008). In order to determine whether the up-regulation of the PD9 gene is related to enhanced defense or survivorship, knockout and overexpressing mutants could be exposed to infection from pathogens, herbivory, and other forms of stress. If overexpression of this gene led to a decrease in infection rates or an increase in resistance to stress, one could conclude that this gene is important in defense and stress tolerance.

The expression of PtD5a was slightly lower in DD-treated cultures than in the solvent control. Again, the use of a greater number of biological replicates would likely give a clearer picture of the effect of decadienal on the expression of this gene. Nevertheless, the decrease in expression of PtD5a aligns with the findings of Alonso et al. (2000) on changes in lipid composition under stress. In this study, 20:5 fatty acids decreased 10% in nitrogen-starved cultures. However, the reason for this decrease could be that 20:5 fatty acids are being released for oxylipin production, and not because the PtD5 gene is down-regulated. As in the case of PD9, a reverse genetics approach could reveal whether this gene is vital to defense or survival under stress.

LIPID METABOLISM GENES

Finally, the expression of the patatin-like phospholipase gene was strongly down-regulated in response to DD, which is in contrast to expected results. Stress has been shown to elicit the release of fatty acids from membrane lipids for oxylipin production, so it was hypothesized that expression of this phospholipase gene would be increased. In addition, Mus et al. (2013) found that all but one of the six phospholipase genes investigated in their study were up-regulated under pH stress, and all of the phospholipases studied were up-regulated under nitrate-limited conditions. The patatin-like phospholipase gene described in this study was not one of the six phospholipase genes examined by Mus et al. (2013), so a specific comparison between the findings of these two studies cannot be made. The collection of data at multiple time points might reveal whether this gene is up-regulated at any time after DD exposure. Oxylipin production has been shown to be a rapid response to herbivory, so the analysis of gene expression at earlier time points such as 30 minutes or 1 hour could show different results than those obtained for the 6-hour time point (Pohnert, 2002). Additionally, the effect of multiple concentrations of DD should be tested, because response to this oxylipin has been shown to be concentration-dependent (Vardi et al., 2006).

Many of the findings in this study were inconclusive or not shown to be statistically significant. Nonetheless, it can be concluded that DD-induced stress had an effect on the expression of some lipid-related genes in this *P. tricornutum*. It is important that investigation into lipid biosynthesis and metabolism processes in microalgae continue, because these organisms could become a suitable feedstock for biodiesel production (Chisti, 2007). Further studies of gene expression related to these processes would give a more complete picture of how these pathways are regulated, and how they can be optimized for high-value lipid production.

Chapter 6: Applications to Practice

The experiences that I had while completing this research have many applications to teaching high school science. Being in the role of the student again reminded me of the anxiety and confusion that can occur when learning something new. There were several moments in which I doubted myself or felt nervous about completing a procedure successfully. When I return to my classroom, I will have renewed compassion for my students as they go through the learning process. As I completed my research, I struggled with many of the same subjects that my students do. As a result, I had several realizations about strategies that I could use to help my students better understand science. For one, my hands-on experiences in the lab equipped me with numerous examples and analogies that I can refer to when explaining science concepts. Additionally, I learned to use technologies important in many areas of science, such as the NCBI genome database, that could be directly incorporated into biology lessons. Last, I discovered, through my own struggle with nomenclature, some strategies that may help students learn chemical formulas and names.

Over time, it is easy for a person not working directly in a research setting to forget why certain concepts are important and how they are used. This experience helped me see how many of the concepts that I teach in biology and chemistry are used directly by scientists working in the field. Dilutions, for example, were an important part of my daily activities in the lab. Cultures of *P. tricornutum* had to be diluted every few days to ensure that appropriate cell counts were maintained, and serial dilutions were required for primer standardization. I struggled at first to complete the calculations necessary for these dilutions, especially since they involved units of measurement that I was not accustomed to using. However, with the help of a patient coach, I eventually gained confidence in my

ability to complete these mathematical procedures. I will now have several examples to refer to when explaining the concepts of molarity and dilutions, and a better understanding of the difficulties students might have with them. For example, a useful way of explaining how to set up a dilution calculation might be, "Start with the type of solution you want". For me, this meant the cell density (or concentration) and volume of *P. tricornutum* culture that I needed. Step two of the problem-solving process could be explained as, "Then, plug in what you have to work with. Put an 'x' next to that value". When performing dilutions of algal cultures, what I had to work with was the cell count of the culture that I currently had, and 'x' represented how much of that culture I should use to make my new solution. Approaching dilutions in this way, with specific examples and processes to use when solving problems, might help students build a better framework for understanding this concept.

It is important to note that in addition to helping me develop strategies for teaching science concepts, this experience reminded me that hands-on experiences are crucial to the learning process. When teaching a unit such as solutions in high school chemistry, labs and kinesthetic activities can be left out of the curriculum if there is pressure to cover a large number of concepts in a short period of time. This experience reminded me that these kinds of experiences are vital to the science classroom, because without them, the learning that is occurring is cursory, and unlikely to persist. Until I actually completed the techniques that I had read about prior to working in the lab, I did not fully understand how these procedures were carried out or how they applied to the overall idea of my experiment. Similarly, students in a high school chemistry class cannot truly understand the meaning of molarity and dilution calculations if they do not actually make solutions or carry out a dilution. The experiences I had while working in the lab renewed my resolve to include these and other types of hands-on experiences in my

curriculum as often as possible, because they help students develop a more thorough understanding of science concepts.

One of the technologies that I used extensively throughout this process could be directly applied to high school biology lessons. The National Center for Biotechnology Information (NCBI) website at http://www.ncbi.nlm.nih.gov/ hosts a collection of databases, articles, and much more that can be easily accessed by anyone with a computer and internet access. This resource makes it easy to search for gene sequences and quickly compare sequences among many types of organisms. One of the features of the NCBI website is a search engine called "BLAST", or Basic Logical Alignment Search Tool. The BLAST function allows one to compare a nucleotide or protein sequence to sequences from every other organism with a sequenced genome. NCBI BLAST is easy and fun to use, because the interface is user-friendly, and the results are rapidly-generated and visual in nature. Figure 7 is an example of an alignment that I completed using the NCBI BLAST tool.

I believe that this technology could be very engaging and informative for students studying DNA, genetics, evolution, and biotechnology. These topics can be difficult to teach because they are somewhat abstract, and require technology that is prohibitively expensive. With some guidance, I believe students could use the NCBI databases and the Basic Logical Alignment Search Tool to explore ideas like homology, DNA structure, and evidence of evolution. For example, students could be given known genes to search for, and then be asked to compare these genes to those from other organisms using the BLAST function. Then, students could explore the results and write or talk about which species have genes that are most homologous to the given gene. Students might be surprised to find that genes in seemingly primitive organisms can have homology to

genes in more complex organisms such as humans. This idea could be further expanded into discussions of evolution and common ancestry.

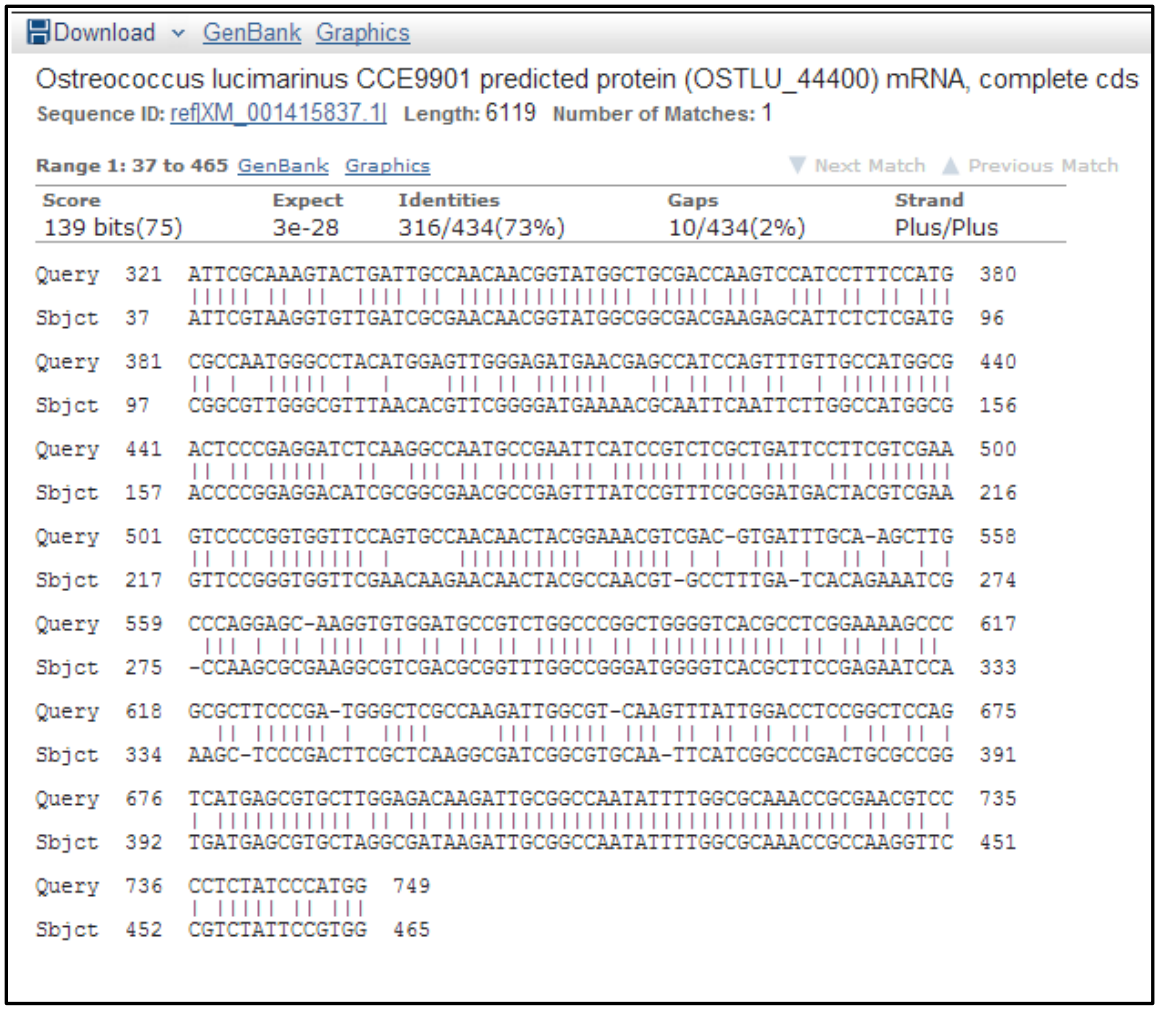


Figure 7: Example of a BLAST query, reproduced with permission from NCBI

The ease with which this tool can search the genomes of many different organisms demonstrates the power of current biotechnology resources. With the help of an enthusiastic teacher, this resource could be used to help students understand the

capabilities of current biotechnology tools, and prompt them to think more deeply about topics like genetics and evolution.

A concept that proved to be a struggle for me at times while completing my research was chemical nomenclature. Nomenclature is a notoriously difficult topic for many high school chemistry students, and the same was true for me as I researched biological pathways in *P. tricornutum*. I often spent as much time researching the meanings of different lipid names as I did the pathways they were involved in. Eventually, I learned that there were several systems for naming lipids, and I found resources that helped me make sense of them. One such resource is the AOCS Lipid Library at http://lipidlibrary.aocs.org/index.html. This online library of lipid-related information was an invaluable resource to me throughout the research process. It describes complex lipids and their synthesis in a visual and understandable manner. This resource, along with a chart of lipid nomenclature that I found at http://biowiki.ucdavis.edu/Biochemistry/Lipids/LIPID_STRUCTURE led me to have an "ah-ha" moment about chemical nomenclature. The chart at the UC Davis biowiki website displayed the names and symbols for lipids in a very ordered way.

Once I saw the nomenclature organized this way, and the visual representations at the Lipid Library, my understanding of lipid nomenclature was greatly increased. I realized that I needed to connect the symbols, names, and pictures of the chemicals together in order to internalize the meanings of them. Table 2 is a re-creation of a table that I constructed to use as a reference while researching lipid biosynthesis pathways.

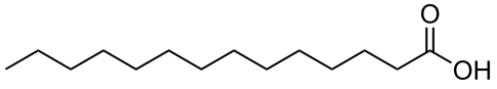
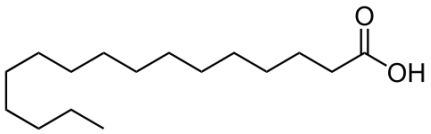
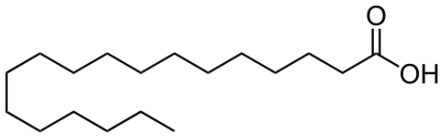
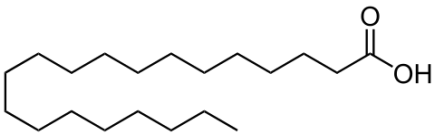
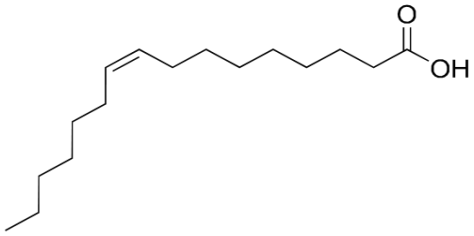
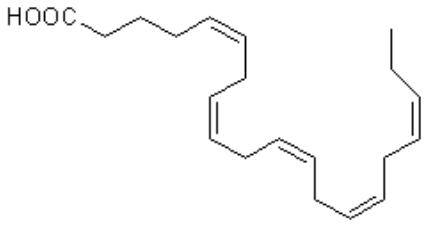
Symbol	Common Name	Systematic Name	Structure
14:0	Myristic acid	Tetradecanoic acid	
16:0	Palmitic acid	Hexadecanoic acid	
18:0	Stearic acid	Octadecanoic acid	
20:0	Arachidic acid	Eicosanoic acid	
$16:1^{\Delta 9}$	Palmitoleic acid	Hexadecenoic acid	
$20:5^{\Delta 5,8,11,14,17}$	EPA	Eicosapentaenoic acid	

Table 2: Graphic organizer of lipid nomenclature and structure (images from commons.wikimedia.org)

I believe that chemistry students could use a similar graphic organizer to more thoroughly understand chemical nomenclature. Students could create the organizers in

their class notebooks as they learned new chemical names, formulas, and structures, and reference them as needed throughout the year. Several different types of learners, such as visual, linguistic, and logical-mathematical, could potentially benefit from this strategy. In addition to helping students learn about chemical nomenclature, strategies like graphic organizers can also help students learn skills that are applicable to learning in general. The ability to effectively organize and systematize information is one that will serve students no matter what field they pursue.

This process helped me see that it is important for teachers to take the role of the student as often as possible. It is also important for educators to get hands-on experience in the fields that they teach. Working in a molecular biology lab brought to life many of the concepts that I teach on a daily basis. I was reminded of the importance of many science concepts like experimental controls, precise measurements, and problem-solving. In a world of ever-increasing competition, it is important that our educators be well-trained in the content they teach and effective strategies for teaching it. This program made me a stronger teacher in many ways, and I believe that similar programs should be made more accessible to teachers around the country. If they were, educators would be able to increase their content knowledge and pedagogical expertise, and as a result, our citizens would be better equipped to compete in today's global economy.

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