EFFECTS OF TEMPERATURE ON DESATURASE ENZYME GENE EXPRESSION IN Brassica napus LEAVES

by

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A thesis submitted in conformity with the requirements for the degree of M.Sc.
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Effects of temperature on desaturase enzyme gene expression in *Brassica napus* leaves.

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**ABSTRACT**

A generally accepted characteristic of higher plants is their ability to adjust the fatty acid composition of their membranes in response to changing growth temperatures. Increasing the levels of *cis*-unsaturated fatty acids increases membrane fluidity, which, as a result, ensures the efficient functioning of the membranes and/or protection of the membrane at low temperatures. Conversely, growth at high temperature results in a decrease in polyunsaturated fatty acids. Such a decrease is associated with reduced membrane fluidity.

*Brassica napus* plants were used to study the effects of temperature stress on desaturase enzyme gene expression. Through the use of northern blot techniques, the mRNA transcript levels from specific cytosolic and chloroplastic desaturase genes were examined. *B. napus* plants grown at 20°C and shifted to 30°C showed only a slight decrease in *fad6* mRNA transcript accumulation, but plants acclimated at 5°C, and shifted to 30°C showed a significant decrease in *fad6* mRNA transcript accumulation. Plants grown at 5°C showed a significant increase in *fad6* mRNA transcript accumulation in comparison to plants grown at 20°C. Results show some correlation of *fad6* gene expression at low temperature, but little or no correlation above 20°C. There were no detectable changes in the expression patterns of *fad2* genes at any of the
temperatures tested, suggesting little or no correlation between \textit{fad2} gene expression and temperature. The results may suggest that chloroplastic and cytosolic desaturase enzymes may utilize different mechanisms to regulate the expression of their genes in response to changes in temperature.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of figures</td>
<td>i</td>
</tr>
<tr>
<td>List of tables</td>
<td>iii</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>Major plant glycerolipids</td>
<td>1</td>
</tr>
<tr>
<td>Fatty acid biosynthesis</td>
<td>2</td>
</tr>
<tr>
<td>Glycerolipid biosynthesis</td>
<td>6</td>
</tr>
<tr>
<td>Prokaryotic pathway</td>
<td>8</td>
</tr>
<tr>
<td>Eukaryotic pathway</td>
<td>8</td>
</tr>
<tr>
<td>Temperature stress</td>
<td>9</td>
</tr>
<tr>
<td>Membrane fluidity and temperature</td>
<td>10</td>
</tr>
<tr>
<td>Plant fatty acid desaturases</td>
<td>11</td>
</tr>
<tr>
<td>Desaturase characterization, isolation, and similarities</td>
<td>11</td>
</tr>
<tr>
<td>Cyanobacterial desaturase enzymes</td>
<td>14</td>
</tr>
<tr>
<td>Characterization of plant fatty acid desaturases</td>
<td>14</td>
</tr>
<tr>
<td>\textit{fad2} mutant ((\omega-6) cytosolic)</td>
<td>15</td>
</tr>
<tr>
<td>\textit{fad3} mutant ((\omega-3) cytosolic)</td>
<td>15</td>
</tr>
<tr>
<td>\textit{fad4} mutant (\textit{trans-}(\Delta^3))</td>
<td>16</td>
</tr>
<tr>
<td>\textit{fad5} mutant ((\omega-9) chloroplastic)</td>
<td>16</td>
</tr>
<tr>
<td>\textit{fad6} mutant ((\omega-6) chloroplastic)</td>
<td>17</td>
</tr>
<tr>
<td>\textit{fad7/8} mutant ((\omega-3) chloroplastic)</td>
<td>17</td>
</tr>
<tr>
<td>Phases of fatty acid desaturation</td>
<td>18</td>
</tr>
<tr>
<td>\textit{Brassica napus}</td>
<td>19</td>
</tr>
<tr>
<td>Regulation of gene expression</td>
<td>19</td>
</tr>
<tr>
<td>Research objectives</td>
<td>20</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>22</td>
</tr>
<tr>
<td>Materials</td>
<td></td>
</tr>
<tr>
<td>Plant material and growth conditions</td>
<td>22</td>
</tr>
<tr>
<td>Stress conditions</td>
<td>22</td>
</tr>
<tr>
<td>Reston variety</td>
<td>22</td>
</tr>
<tr>
<td>Mutant variety</td>
<td>23</td>
</tr>
<tr>
<td>Methods</td>
<td></td>
</tr>
<tr>
<td>RNA extraction</td>
<td>23</td>
</tr>
<tr>
<td>Quantitation and quality of total RNA</td>
<td>24</td>
</tr>
<tr>
<td>cDNA probe preparation</td>
<td>24</td>
</tr>
<tr>
<td>\textit{E. coli} transformation</td>
<td>24</td>
</tr>
<tr>
<td>Competent cells</td>
<td>24</td>
</tr>
<tr>
<td>Transformation</td>
<td>25</td>
</tr>
</tbody>
</table>
Plasmid DNA isolation
(midi prep DNA purification)

PCR reactions
  Primer design
  PCR

Restriction endonuclease digestion
DNA gel electrophoresis
  Low melting agarose
RNA gel electrophoresis and northern blot hybridization

RESULTS

Probe preparation
Primer design and PCR reactions
Low melting agarose gel
Regulation of cytosolic desaturase enzyme gene expression
  Effect of high temperature on fad2 gene expression
    Differences between plants grown at 5°C and 20°C, and shifted to 30°C
  Effect of low temperature on fad2 gene expression
    B. napus plants
    HL 29 and HL 35 plants
Regulation of chloroplastic desaturase enzyme gene expression
  Effect of high temperature on fad6 gene expression
    Differences between plants grown at 5°C and 20°C, and shifted to 30°C
  Effect of low temperature on fad6 gene expression
    Comparison of fad2 and fad6 gene expression in HL29 and HL35 plants
Regulation of fad7/8 gene expression
  Effect of high temperature on fad7/8 gene expression

DISCUSSION

fad6 versus fad2 gene expression
fad2 gene expression
fad6 gene expression
fad7/8 gene expression
Desaturase enzyme activity
HL29 and HL35
Conclusion
Future studies

REFERENCES
LIST OF FIGURES

Figure 1. The major diacylglycerols in plant membranes. 3

Figure 2. The pathways for the synthesis of chloroplast lipids in 16:3-plants. 7

Figure 3. 16:3-fatty acid showing the number and position of carbon: carbon double bonds. $\Delta^n$ denotes the position of the double bond on the carbon atom from the COOH end, $\omega_n$ denotes the position of the double bond on the carbon atom from the CH$_3$ end of the fatty acid and fatty acid desaturases in plants. 12

Figure 4. Regulation of gene expression in eukaryotic organisms. 21

Figure 5. PCR reaction mixture products were run on a 0.7% low melting agarose gel stained with EtBr to isolate amplified sequences. A 5 $\mu$L sample of each reaction mixture was run to verify the brand of interest. 35

Figure 6. Ethidium bromide staining of 7.5 $\mu$g of total RNA loaded per lane and RNA gel-blot analysis of $\omega$-6 cytosolic gene transcript accumulation during high and low temperature exposure (plants were grown at 20°C and shifted to 30°C from 0 to 7 days; 20-30, and plants were grown at 20°C and shifted to $5^\circ$C from 0 to 21 days; 20-5). 37

Figure 7. Ethidium bromide staining of 7.5 $\mu$g of total RNA loaded per lane and RNA gel-blot analysis of $\omega$-6 cytosolic gene transcript accumulation during low and high temperature exposure (plants were grown at 20°C for 3 weeks, shifted to $5^\circ$C for 4 weeks, and then shifted to 30°C). Total RNA was extracted at 30°C from 0 to 120 hours. 39

Figure 8. Ethidium bromide staining of 7.5 $\mu$g of total RNA loaded per lane and RNA gel-blot analysis of $\omega$-6 cytosolic gene transcript accumulation during low and high temperature exposure (plants were grown at $5^\circ$C, and at 20°C). 43

Figure 9. Ethidium bromide staining of 10 $\mu$g of total RNA loaded per lane and RNA gel blot analysis of $\omega$-6 chloroplastic gene transcript accumulation during high temperature exposure (plants were grown at 20°C, and then shifted to 30°C for seven days). 45
Figure 10. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane and RNA gel-blot analysis of ω-6 chloroplastic gene transcript accumulation during low and high temperature exposure (plants were grown at 20°C for three weeks, shifted to 5°C for 4 weeks, and then shifted to 30°C). Total RNA was extracted at 30°C from 0 to 120 hours.

Figure 11. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane and RNA gel-blot analysis of ω-6 chloroplastic gene transcript accumulation during low and high temperature exposure (plants were grown at 5°C, and at 20°C).

Figure 12. Ethidium bromide staining of 10 μg of total RNA loaded per lane and RNA gel-blot analysis of ω-3 chloroplastic gene transcript accumulation during high temperature exposure (plants were grown at 20°C and then shifted to 30°C for seven days).
LIST OF TABLES

Table 1. Oligomers obtained using DNASIS sequence analysis program, showing the GC content percentages, melting temperatures, and sequences, of the oligomers. These oligomer sequences were constructed by Gibco BRL Protogene, for subsequent use in PCR reactions as primers.
LIST OF ABBREVIATIONS

ACCase: acetyl-coenzyme A carboxylase
ACP: acyl carrier protein
ATP: adenosine triphosphate
BCCP: biotin carboxyl carrier protein
BLD: basal level desaturation
C-n: carbon length of fatty acids
CoA: coenzyme A
DAG: diacylglycerol
des A, B, C, D: desaturase A, B, C, D
DGDG: digalactosyldiacylglycerol
EtBr: ethidium bromide
fad2: locus which codes for cytosolic ω6-desaturase gene
fad3: locus which codes for cytosolic ω3-desaturase gene
fad4: locus which codes for cytosolic trans-Δ3 gene
fad5: locus which codes for chloroplast ω9-desaturase gene
fad6: locus which codes for chloroplast ω6-desaturase gene
fad7, fad8: loci which code for chloroplast ω3-desaturase gene
HL: high linolenate
KASII: 3-ketoacyl-ACP synthase II
kDa: kilo-daltons
LMA: low-melting agarose
LTD: low temperature-induced desaturation
MGDG: monogalactosyldiacylglycerol
NADH: reduced nicotinamide adenine dinucleotide
NADPH: reduced nicotinamide adenine dinucleotide phosphate
PA: phosphatidic acid
PC: phosphatidylcholine
PCR: polymerase chain reaction
PE: phosphatidylethanolamine
PG: phosphatidylglycerol
PI: phosphatidylinositol
sn: stereospecific numbering
ωn: nth carbon from the methyl end of a fatty acid chain
Δn: nth carbon from the carboxyl group of a fatty acid chain
16:0: palmitic acid
18:0: stearic acid
18:1: cis 9-oleic acid
18:2: cis 9, 12-linoleic acid
α18:3: cis 9, 12, 15-linolenic acid
INTRODUCTION

Higher plants are incapable of removing themselves from their environments, as a result, mechanisms have evolved allowing them to acclimate to suboptimal conditions. Temperature is one of the most important factors in the environment of plants. Membrane fluidity is crucial to proper and efficient functioning of membrane bound proteins and is considered to play an important role in the survival of plants at low temperatures. Plants can maintain the level of molecular motion or fluidity of their membrane lipids by regulating the number of double bonds in the fatty acids of their lipids. When the fluidity of their membrane lipids is reduced, due to decreases in temperature, additional double bonds are introduced into the fatty acids of their lipids. This results in a restoration in membrane fluidity. Desaturase enzymes are responsible for the introduction of these specific double bonds. The purpose of this thesis was to examine the expression of desaturase genes, in order to determine if regulation of gene expression is part of the process involved in regulating membrane fluidity.

LITERATURE REVIEW

Major Plant Glycerolipids

The major plant lipids are diacylglycerols, which are structures consisting of two fatty acids esterified to the sn-1 and sn-2 positions [IUPAC-IUB
Commission on Biochemical Nomenclature, “stereospecific numbering” (sn system) of a glycerol backbone (Harwood, 1980). Diacylglycerols can be classified into two groups: phospholipids, which are the major components of cytosolic membranes; and glycolipids, which are major components of chloroplastic membranes (see Figure 1).

Phospholipids and glycolipids consist of a phosphate and a sugar head group attached to the sn-3 position of a glycerol backbone, respectively. The other structural components of phospholipids and glycolipids consist of fatty acyl chains esterified to the sn-1 and sn-2 positions of glycerol backbones. The major cytosolic phospholipids in plants are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS). The chloroplast glycolipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). In addition, the chloroplast contains the phospholipid PG.

**Fatty Acid Biosynthesis**

In plants the formation of the major fatty acids; palmitate (16:0), oleate (18:1), linoleate (18:2), and α-linolenate (18:3) require the assembly of long chains of carbon atoms through the combined activities of acetyl-CoA carboxylase and fatty acid synthase. The acetyl-CoA may be supplied by at least two pathways, depending on the tissues studied (Harwood, 1996). In chloroplasts, acetyl-CoA can be supplied by a plastidial pyruvate
Figure 1. The major diacylglycerols in plant membranes (FA=fatty acid).
decarboxylase/dehydrogenase complex. Acetyl-CoA can also be supplied by a mitochondrial pyruvate/dehydrogenase complex which produces free acetate, where the movement of the latter into the chloroplast occurs thereby regenerating acetyl-CoA by a chloroplast acetyl-CoA synthase enzyme (Harwood, 1996).

Acetyl-CoA carboxylase (ACCase) carries out the first step in fatty acid biosynthesis (Harwood, 1996; Markham et al., 1997; Browse and Somerville, 1991). This is a soluble Class 1 biotin-containing enzyme that requires ATP and catalyses the formation of malonyl-CoA from acetyl-CoA and bicarbonate (Harwood, 1996; Burton and Rawsthorne, 1997). It is believed that the carboxylation of acetyl-CoA to malonyl-CoA is the rate limiting step in fatty acid synthesis (Markham et al., 1997; Browse and Somerville, 1991).

In plants, the ACCase enzyme is a single polypeptide containing three functional domains; biotin carboxylase, carboxyl carrier protein (which binds a biotin prosthetic group), and carboxyl transferase. ACCase catalyses its reaction in two main steps on its two physically distinct catalytic sites. The first partial reaction that occurs involves the use of ATP to carboxylate the biotin prosthetic group of biotin carboxyl carrier protein (BCCP). The second partial reaction involves the action of a carboxyltransferase where a carboxyl group is transferred to acetyl-CoA to yield malonyl-CoA (Harwood, 1996).

The next series of reactions involved in fatty acid synthesis are catalyzed by a multienzyme complex, known as fatty acid synthase. An abundant component of fatty acid synthase is known as Acyl Carrier Protein, which is
required by a host of enzymes including acyltransferases (Harwood, 1996). In plant cells, most of the steps of fatty synthesis and elongation involve reactions which require ACP. ACP acts as a cofactor and serves to carry the acyl chain from one enzyme's active site to the next.

Malonyl-CoA is transferred to ACP by a soluble tranacylase, resulting in malonyl-ACP. Malonyl-ACP is then utilized in a series of condensation reactions. The reaction of malonyl-ACP with acetyl-ACP is catalysed by β-ketoacyl-ACP I, which is the initial condensation reaction for fatty acid synthesis (Harwood, 1996). Each cycle in fatty acid synthesis is initiated by the condensation of a fatty acyl group (which is linked by a thioester bond to the active site of 3-ketoacyl-ACP synthase) with malonyl-ACP to produce a 3-ketoacyl-ACP and CO₂. The cycle continues with the sequential action of 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP reductase to form a new acyl chain that is two carbons longer than at the start of the cycle (Browse and Somerville, 1991).

Each successive round of condensation reactions, each utilising a molecule of malonyl-ACP, followed by keto reduction, dehydration and enoyl reduction, results in the formation of palmitoyl-ACP (16:0-ACP; where 16 is the carbon chain length and 0 is the number of double bonds in the fatty acid represented). The 16:0-ACP moiety can either be converted to a free fatty acid and used for the synthesis of glycerolipids inside and outside of the chloroplast or it can be elongated to 18 carbons in length (18:0-ACP).
The enzyme responsible for carrying out the elongation of 16:0-ACP to stearoyl-ACP (18:0-ACP) is 3-ketoacyl-ACP synthase II (KAS II) (Harwood, 1996). Once 18:0-ACP is formed by the elongation of 16:0-ACP, 18:0-ACP becomes the substrate for the introduction of the first cis-double bond. The formation of the double bond is mediated by a stearoyl-ACP desaturase to form oleoyl-ACP (18:1-ACP) (Harwood, 1996). As a result, 16:0-ACP and 18:1-ACP are the main products of plastid fatty acid synthesis.

**Glycerolipid Biosynthesis**

The initial step in glycerolipid biosynthesis occurs in the plastid with the production of 16:0-ACP by fatty acid synthase. A major portion of 16:0-ACP is elongated to 18:0-ACP and then desaturated to 18:1-ACP by a soluble desaturase (Falcone et al., 1994). These products when formed, enter into one of two distinct pathways in leaf cells for the biosynthesis of membrane glycerolipids and the associated production of polyunsaturated fatty acids (Roughan et al., 1980; Browse and Somerville, 1991; McConn et al., 1994) (see figure 2). The prokaryotic pathway is initiated by the acylation of glycerol-3-phosphate within the plastid. The reactions of the eukaryotic pathway take place in the endoplasmic reticulum following the export of 16:0 and 18:1 fatty acids from the plastid and conversion of CoA esters in the cytoplasm (Falcone et al., 1994).
Figure 2. The pathway for the synthesis of chloroplast lipids in 16:3-plants.
Prokaryotic Pathway

The prokaryotic pathway (Roughan et al., 1980; Miquel and Browse, 1992) located in the inner chloroplast envelope uses 18:1-ACP and 16:0-ACP for the sequential acylation of sn-glycerol-3-phosphate. Due to the substrate specificities of the chloroplast acyltransferases (Frentzen et al., 1983; Miquel and Browse, 1992) the phosphatidic acid (PA) formed has 18:1 at the sn-1 position and 16:0 at the sn-2 position. This PA is used directly for the synthesis of phosphatidylglycerol (PG) and, via diacylglycerol (DAG), as a precursor for the synthesis of the other major chloroplast lipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQ) (Miquel and Browse, 1992; Browse and Somerville, 1991).

Eukaryotic Pathway

The eukaryotic pathway begins with the hydrolysis of 16:0-ACP and 18:1-ACP followed by the export of these free fatty acids through the two membranes of the chloroplast envelope, and their conversion to CoA thioesters by an acyl-CoA synthetase in the outer envelope. The CoA esters are used for the synthesis of 16:0/18:1- and 18:1/18:1-PA mainly in the endoplasmic reticulum (Miquel and Browse, 1992; Browse and Somerville, 1991). However, recent evidence (Imperial, M.Sc. thesis, and Williams, personal communications) suggests that PC may be synthesized from partially unsaturated fatty acids. The acyl-CoA-specific acyltransferases of the endoplasmic reticulum only produce PA with an 18-carbon fatty acid at the sn-2 position; 16:0, when present, is at the
sn-1 position. This PA gives rise to the phospholipids, including; phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), which are characteristic of extrachloroplast membranes (Miquel and Browse, 1992). In addition the DAG moiety of PC may be returned to the chloroplast envelope where it contributes to the production of thylakoid lipids (Roughan and Slack, 1982; Miquel and Browse, 1992).

**Temperature Stress**

Plants are incapable of escaping from changing environments. In order for plants to survive unfavorable conditions they have evolved mechanisms for acclimating to temperature stress. Low temperature is one of the more important environmental factors which limit plant growth, distribution, survival, and crop yields worldwide (Gray et al., 1997; Kodama et al., 1994). Exposure to low, nonfreezing temperatures can induce genetic, morphological, and physiological changes in plants (Gray et al., 1997). Low temperatures have also been shown to induce a number of alterations in cellular components, including the extent of fatty acid unsaturation, the composition of glycerolipids, the positional redistribution of saturated and unsaturated fatty acids within lipid molecules, changes in protein composition, and activation of ion channels (Johnson and Williams, 1989; Murata and Los, 1997).

Biochemical and molecular analyses have shown that during the induction of freezing tolerance certain genes are differentially expressed and specific proteins are accumulated (Gray et al., 1997). Cold-induced genes have been
isolated and characterized in many species and there is evidence which shows a high correlation between the expression of these cold-inducible genes (which appear to be up-regulated by low temperature), and the development of freezing tolerance (Gray et al., 1997). However, the mechanisms by which low temperature signals are perceived and transduced into specific biochemical responses is largely unknown.

Membrane Fluidity and Temperature

Polyunsaturation of fatty acids is considered to be a key factor in the adaptation to low temperatures in higher plants (Gibson et al., 1994; Johnson and Williams, 1989; Kodama et al., 1994; Lee and Guerra, 1994; Murata and Los, 1997; Somerville and Browse, 1991; Williams et al., 1988; Williams et al., 1992). The term “fluidity” is used to describe the extent of disorder and the molecular motion within a lipid bilayer. Fluidity affects movement across membranes, the integrity of the membrane, and such membrane bound processes as electron transport (Williams et al., 1988). The extent of unsaturation of membrane lipids is the major factor which influences the fluidity of membrane lipids (Demandre et al., 1986, Murata and Los, 1997, Williams et al., 1988).

A decrease in temperature initially leads to a decrease in membrane fluidity, and eventually, as a result of this decrease in membrane fluidity, an increase in the level of unsaturated fatty acids results (Murata and Los, 1997; Williams et al., 1988; Williams et al., 1992). It is generally believed that an
increase in the unsaturation of fatty acids provides organisms with some degree of protection from low temperature damage by eventually restoring membrane fluidity (Murata and Los, 1997; Williams et al., 1988; Williams et al., 1992).

**Plant Fatty Acid Desaturases**

The number, position, and stereochemical orientation of carbon:carbon double bonds is critical to the biological activity of certain fatty acids (see figure 3 A). The enzymes which are responsible for introducing carbon:carbon double bonds into fatty acids are termed fatty acid desaturase enzymes. Plant fatty acid desaturases require molecular oxygen, NADH via cytochrome b₅, or NADPH and ferredoxin to carry out their reactions (Mazliak, 1994). Fatty acid desaturases are important in the regulation of the degree of unsaturation of membrane glycerolipids and, in turn, the ability of certain organisms to tolerate low temperatures (Murata et al., 1995, p 3, Shanklin et al., 1997). The fatty acid desaturases in plants include: acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases; which introduce double bonds into fatty acids esterified to CoA, ACP, and glycerolipids respectively (Murata and Los, 1997).

**Desaturase Characterization, Isolation, and Simmilarities**

Plant fatty acid desaturases fall into one of two classes. The first, the soluble class is found in the plastids of plants. The only plant desaturase that has been characterized in any detail is the soluble stearoyl-ACP desaturase which inserts a double bond at the ω-9 position in 18:0-ACP (Browse et al.,
Figure 3 A. A 16:3-fatty acid showing the number and position of carbon:carbon double bonds. \( \Delta^n \) denotes the position of the double bond on the carbon atom from the COOH end and \( \omega \) denotes the position of the double bond on the carbon atom from the \( \text{CH}_3 \) end of the fatty acid. B. Fatty acid desaturases in plants (fab = fatty acid biosynthesis, fad = fatty acid desaturase).
Stearoyl-ACP desaturase was purified first from safflower, and it was found to be a dimer of 38 kDa subunits, utilising reduced ferredoxin as an electron donor (Harwood, 1996) and oxygen (Mazliak, 1994). The c-DNA of stearoyl-ACP desaturase has been sequenced from several plant species including castor bean, cucumber, soya bean, and spinach (Harwood, 1996; Mazliak, 1994). There appears to be considerable sequence conservation between the stearoyl-ACP desaturases from different higher plants but none with the stearoyl-ACP desaturases of vertebrates or fungi, nor with the cyanobacterial $\Delta^{12}$-desaturase or the cyanobacterial $\Delta^{9}$-desaturase (Harwood, 1996) suggesting that the plant enzyme is independently evolved (Somerville and Browse, 1991).

The second class of desaturases are integral membrane proteins. This class of desaturases are less characterized due to difficulties in isolating sufficient quantities of protein from natural sources to perform biophysical characterizations (Shanklin et al., 1997). Several membrane-bound desaturases have been cloned from plants and cyanobacteria (Schmidt et al., 1995; Hitz et al., 1994; Nishiuchi et al., 1994; Reddy et al., 1993). Comparisons of the deduced amino acid sequences have revealed regions of homology which are highly conserved in membrane-bound desaturases from plants, cyanobacteria, yeast and mammals. Through comparisons of amino acid sequences of desaturase genes from GenBank, Shanklin et al. found consensus histidine-containing motifs amongst 17 membrane-bound fatty acid desaturases, two membrane bound bacterial hydroxylases, and one hemoglobin (Shanklin et al., 1995).
**Cyanobacterial Desaturase Enzymes**

Cyanobacteria can be classified into four groups with respect to the mode of desaturation of fatty acids. Strains in the first group can desaturate only at the Δ⁹ position. The second are characterized by the ability to introduce double bonds at the Δ⁹, Δ¹₂, and ω-3 positions. The third desaturate at the Δ⁶, Δ⁹, and Δ¹₂ positions. Finally the fourth group desaturate at the Δ⁶, Δ⁹, Δ¹₂, and ω-3 positions (Sakamoto et al., 1994). Each of the desaturases are encoded by a single-copy gene, designated desD, desC, desA, and desB respectively (Murata and Los, 1997). All desaturases in cyanobacteria that have been studied to date are of the acyl-lipid type. These desaturases are located in both the thylakoid and plasma membranes of cyanobacterial cells (Murata and Los, 1997).

**Characterization of Plant Fatty Acid Desaturases**

Much of what is known about plant desaturases has come from the characterization of a series of *Arabidopsis thaliana* mutants that have been shown to have defects in fatty acid desaturation. To date there have been seven mutants discovered that lack or are deficient in certain desaturases. The availability of these mutants has been useful in elucidating the biochemistry of fatty acid synthesis. Most of these mutations cause a loss or reduction in the amount of an unsaturated fatty acid and the corresponding accumulation of a less unsaturated precursor (Somerville and Browse, 1991). The fatty acid desaturases in plants and their reactions are illustrated in figure 3 B.
**fad2 Mutant (ω-6 cytosolic)**

Mutants of *A. thaliana* at the *fad2* locus are deficient in activity of the endoplasmic reticulum desaturase (Miquel and Browse, 1992; Okuley *et al.*, 1994). The enzyme responsible for the synthesis of linoleate (18:1 to 18:2) is the plant oleate desaturase of the endoplasmic reticulum. In *A. thaliana* the activity of the oleate desaturase was found to be controlled by the *fad2* locus (Miquel and Browse, 1992; Okuley *et al.*, 1994). The *fad2* allele was identified from a pool of *A. thaliana* plants in which mutations were created by T-DNA insertion by determining the fatty acid compositions of root, leaf, and seed tissues in which there were increased levels of oleate (Okuley *et al.*, 1994).

**fad3 Mutant (ω-3 cytosolic)**

The *fad3* allele was also identified from a pool of *A. thaliana* plants in which mutations had been generated by T-DNA insertion. The overall composition of leaf and root lipids from a mutant of *A. thaliana* were characterized which revealed reduced levels of linolenate (18:3) and increased levels of linoleate (18:2). Comparisons of fatty acid compositions of lipids from wild type and mutant plants revealed that the chloroplast lipids were not significantly affected by the mutation. However, each of the phospholipids synthesized in the endoplasmic reticulum in the mutant showed a significant reduction in 18:3 in comparison to wild type. These results revealed that *fad3*
mutants were deficient in the activity of an endoplasmic 18:2 desaturase (Browse et al., 1993).

**fad4 Mutant (trans-Δ³)**

*fad4* mutants are specifically defective in the accumulation of a *trans*-fatty acid at the *sn*-2 position of phosphatidylglycerol (PG) in the plastid (Somerville and Browse, 1991).

**fad5 Mutant (ω-9 chloroplastic)**

A mutant line, which carries a mutation at the *fad5* locus was isolated from *A. thaliana* which was deficient in the activity of the chloroplast ω-9 desaturase. The function of the chloroplast ω-9 desaturase is to convert palmitic acid (16:0) at position *sn*-2 of MGDG to *cis*-16:1 (Kunst et al., 1989). The result of this mutation was a high accumulation of palmitic acid (16:0), and an overall reduction in the level of unsaturation of chloroplast lipids by the prokaryotic pathway (Somerville and Browse, 1991). Additional *A. thaliana* studies have shown that the insertion of the first *cis*-double bond at the ω-9 position is usually required before other chloroplast desaturases can act on the fatty acid chain (Kunst et al., 1989a).
fad6 Mutant (ω-6 chloroplastic)

The fad6 mutant is deficient in desaturation of 16:1 to 16:2, and 18:1 to 18:2, on all chloroplast lipids. The fad6 mutant line was isolated by using ethyl methanesulfonate to mutate A. thaliana seeds. The biochemical phenotype of the fad6 mutant was characterized by the absence of trienoic fatty acids derived from the prokaryotic pathway and an increased accumulation of 16:1 and 18:1 monounsaturated species (Falcone et al., 1994). Other effects of this mutation were observed on chloroplast ultrastructure, thylakoid membrane protein and chlorophyll content, electron transport rates, and the thermal stability of the photosynthetic membranes (Hugly et al., 1989).

Chloroplastic ω-6 desaturase introduces the second cis-double bond on both 16 and 18 carbon fatty acids, and works on both sn-1 and sn-2 positions of all major chloroplast lipids (Browse et al., 1989).

fad7/8 Mutant (ω-3 chloroplastic)

Clones for both fad7 and fad8 were identified by heterologous screening using the Brassica napus fad3 gene as a probe (Iba et al., 1993). Studies with the fad8 mutant indicated the presence of a cold-induced desaturase that was not expressed at normal growth temperatures. The existence of this enzyme, which appeared to be an isozyme of fad7, became obvious when it was found that three independent fad7 mutants had wild-type fatty acid compositions at low temperatures. However, these three mutants showed a substantial loss of
trienoic fatty acids (16:3 and 18:3) at growth temperatures above 26°C (Somerville and Browse, 1991). This suggested that the role of fad8 desaturase was to provide increased ω-3 desaturase activity in plants that were exposed to low growth temperature (Gibson et al., 1994).

Chloroplastic ω-3 desaturase introduces the third cis-double bond at the ω-3 position of acyl groups of chloroplast membrane lipids (Gibson et al., 1994). This enzyme shows no specificity for fatty acid chain length or point of attachment of fatty acids to the glycerol backbone.

**Phases of Fatty Acid Desaturation**

At low temperatures there exist two phases of fatty acid desaturation. A very rapid phase of desaturation of 16:0 to 16:2 and 18:1 to 18:2, termed; low temperature-induced desaturation (LTD), and a lower level of desaturation, called the normal or basal level of desaturation (BLD) (Williams et al., 1988; Williams et al., 1992a; Williams et al., 1992b; Williams et al., 1993). The LTD response to low temperature has been shown to occur as a result of slow acclimation to growth temperature and not as an immediate response to a change in environmental temperature (Williams et al., 1988; Williams et al., 1992A, ). Studies on Brassica napus, a 16:3-plant, showed that growth at low temperatures significantly increased the rate of desaturation of both chloroplastic MGDG and cytosolic PC lipids (Williams et al., 1992A; Williams et al., 1992B). Further studies revealed that the LTD process was inactivated by exposure to
high temperature, and that LTD was reactivated when plants were transferred to low temperatures but at a much slower rate (Williams et al., 1992B). These results suggest that the desaturation of fatty acids in plants is complex and may be under genetic control.

**Brassica napus**

*B. napus* (a 16:3-plant) was produced as a result of a cross between *Brassica campestris* and *Brassica oleracea*. Since *B. napus* was propagated as the F₁ progeny of a cross between two diploid (2N) species, as previously mentioned, it possesses the equivalent of four genomes, thus is an amphidiploid. *B. napus* seeds contain 40-44% oil on a dry weight basis. Plant oils are of major importance, both economically and nutritionally. Erucic acid, a nutritionally undesirable fatty acid (22:1), was once a major component of the oil extracted from rapeseed. High levels of 18:3 fatty acids in Canola oils are also undesirable, whereas high levels of 18:2 fatty acids are indicative of a high quality oil. Thus breeding programs have focussed on reducing the levels of 22:1 and 18:3 fatty acids, while increasing the level of 18:1 and 18:2 fatty acids in Canola oils.

**Regulation of Gene Expression**

The majority of eukaryotic genes are regulated at the level of transcription. Transcription is regulated through two types of DNA sequences known as promoters and enhancers. Promoter sequences are located close to the start
site of transcription and enhancers are located further from the start site and are able to act over long distances. Both of these elements function through the specific binding of proteins, known as nuclear transcription factors which recognize short motifs within the promoter and enhancer elements (see figure 4). These bound factors are known to either accelerate or retard the rate of transcription initiation of primary transcripts by RNA polymerase II. There are three possible types of interactions which may exist on transcription complexes; protein-DNA, protein-protein, and protein-RNA polymerase II. All of these interactions contribute in mediating the rates of transcription initiation. Other factors involved in the successful processing of a functional gene, include transcription elongation, transcript stability, transcript processing, translation, and posttranslational processing. If the desaturase genes studied thus far are regulated at the level of transcription in response to temperature, then it follows that the steady state levels of transcript accumulation be studied.

**Research Objectives**

This study was designed to determine the effects of temperature stress on desaturase enzyme gene expression in the leaves of different varieties of *B. napus*. This study was also performed to determine if desaturase enzyme activity correlated with desaturase enzyme gene expression at various temperatures. Through the use of northern blot techniques, the mRNA transcript levels from specific cytosolic and chloroplastic desaturase genes were examined in response to temperature stress.
Figure 4. The regulation of gene expression in eukaryotic organisms at the level of transcription.
MATERIALS AND METHODS

MATERIALS

Plant Material and Growth Conditions

Reston and mutant varieties of *Brassica napus* plants were grown from seed in controlled environment growth chambers at 20°C with 16 hours of daylight under mixed incandescent (25 W, GE Canada, Mississauga, ON, Canada)/fluorescent (40 W, F72T12/CWHO, GE Canada) light (approximately 200 μmol m⁻² s⁻¹). The plants were watered every second day, and a nutrient solution was added every seventh day.

Stress Conditions

a) Reston Variety

For the cold acclimation studies, seedlings were grown under the above conditions for four weeks and then transferred to a growth chamber set at 5°C. Leaf samples were taken for subsequent RNA extractions at seven day intervals. For the elevated temperature studies, seedlings were grown under the above conditions for four weeks. The seedlings were then transferred to a growth room set at 5°C for three weeks, and were then transferred to a growth room set at 30°C. Leaf samples were taken at various intervals over a seven day period for RNA extractions.
b) Mutant Variety

Mutant lines of *B. napus* seedlings (HL29, 35) were grown at 20°C under the above conditions. Leaf samples were taken after three weeks for RNA extractions. Mutant lines were also grown at 20°C (as the above conditions) for three weeks and then transferred to 5°C for three weeks. Leaf samples were taken for RNA extractions.

**METHODS**

**RNA Extraction**

Approximately 0.150 g of leaf tissue was removed from each plant and rapidly cooled in liquid nitrogen. Leaf material was ground using a pre-cooled mortar and pestle to a fine powder. Total RNA was isolated from the leaf material using Qiagen's RNeasy Total RNA Plant Extraction Kit. Finely ground powder was transferred to 450 µl of RLT lysis buffer (a buffered aqueous solution containing guanidium isothiocyanate) with 0.45 µl of β-mercaptoethanol and vortexed vigorously. Samples were incubated at 56°C for 3 minutes to help disrupt tissue. The lysate was then transferred to a QIAshredder spin column and centrifuged for 2 minutes to allow for the separation of all cell debris. The flow through fraction was then mixed with 225 µl of 95% ethanol. The mixture was then applied to a RNeasy spin column and centrifuged to trap the RNA in the column. The column was washed with 700 µl of RPE wash buffer and centrifuged. The column was then washed two times with RPE wash buffer with
ethanol and centrifuged. RNA was eluted with 50 μl of RNase-free water. The yield of RNA extracted was expected to be in excess of 30 μg, thus a second elution step was performed. The eluted RNA was stored at -20°C in a sterile 1.5 ml eppendorf tube.

Quantitation and Quality of total RNA

The concentration and purity of RNA extracted was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer. The ratio between the absorbance values at 260 nm and 280 nm gave an estimate of RNA purity. The quantity of RNA extracted was calculated, where an absorbance of 1 unit at 260 nm corresponded to 40 μg of RNA per ml (A_{260} = 1 = 40 μg/ml).

cDNA Probe Preparation

a) E. coli Transformation

Competent Cells

*Escherichia coli* strain DH5α was streaked on to LB plates (10 g/L Bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 15 g/L agar, 1 L deionized H₂O). The plates were incubated at 37°C for 16 hours. One colony was isolated and placed into 5 ml of LB broth (10 g/L Bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 1 L deionized H₂O) in a 15 ml polypropylene tube. The colony was dispersed and incubated overnight at 37°C. Five hundred microliters of the
overnight culture was inoculated into sterile 250 ml flasks containing 50 ml of SOB medium (950 ml deionized H₂O, 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 10 ml of 250 mM KCl, 0.2 ml of 5 N NaOH) for 3-4 hours. These cultures were grown until the OD₆₀₀ of the culture was 0.5. Cells were transferred to a sterile 50 ml polypropylene Falcon tube, chilled on ice for 15 minutes, and centrifuged at 750 g for 15 minutes at 4°C in a Sorval rotor (model SS-34). The media was decanted from the cell pellets, and the pellets were dissolved in 16.5 ml of Competency I buffer (100 ml distilled H₂O, 20 ml of 1 M KCl, 1.2 ml of 5 M potassium acetate, 12 ml of 1 M CaCl₂, 30 ml ultra pure glycerol, 0.2 M acetic acid to pH 5.8), with gentle mixing. The cells were incubated on ice for one hour. Cells were centrifuged at 1050 g in a Sorval rotor (model SS-34), for 15 minutes at 4°C and supernatant was discarded. The pellet was resuspended in 2 ml of Competency Buffer II (100 ml distilled H₂O, 4 ml of 0.5 M MOPS, 2 ml of 1 M KCl, 15 ml of 1 M CaCl₂, 30 ml of ultra pure glycerol, 1 N NaOH for pH 6.8) and mixed gently. The cells were incubated on ice for 15 minutes, cells were aliquoted into microcentrifuge tubes and stored at -70°C.

**Transformation**

Two hundred microliters of competent cells were thawed by hand and placed on ice for 10 minutes. Twenty nanograms of DNA dissolved in water was added to the competent cells and then placed on ice for 30 min. The mixture was heat shocked at 42°C for 45 seconds and chilled on ice for 2 minutes. One milliliter of SOC medium (SOB medium plus 1 M glucose solution) was added to
the mixture and mixed gently. The mixture was incubated at 37°C for one hour and spread-plated onto LB plates supplemented with ampicillin (50 μg/ml) for overnight incubations.

**Plasmid DNA Isolation (Midi Prep DNA Purification)**

Plasmid DNA was isolated and purified using the Wizard Midiprep DNA Purification System from Promega. Fifty milliliter cultures of the transformed *E. coli* were grown in LB broth supplemented with ampicillin (50 μg/ml) overnight. Cells were pelleted by centrifugation at 10000 *g* for ten minutes at 4°C. The cell pellet was completely resuspended in 3 ml of Cell Resuspension Solution (50 mM Tris-HCl; pH 7.5, 10 mM EDTA, 100 μg/ml RNase A). Three milliliters of Cell Lysis Solution (0.2 M NaOH, 1% SDS) was added. The mixture was mixed by gentle inversion. Three ml of Neutralization Solution (1.32 M Potassium acetate, pH 4.8) was added, and immediately mixed with gentle inversions. The bacterial lysate was centrifuged at 14000 *g* for 15 minutes at 4°C. The pellet was discarded, and the lysate was transferred to a 50 ml polypropylene Falcon tube. Ten milliliters of Wizard Resin was added to the lysate and swirled to mix. The resin/DNA lysate mixture was transferred to a Wizard Midicolumn to which a vacuum source was applied. The resin/lysate mixture was washed twice with 15 ml of Column Wash Solution (200 mM NaCl, 20 mM Tris-HCl; pH 7.5, 5 mM EDTA, 95% ethanol) in the column. The resin was dried for 5-10 minutes, by the application of a tap vacuum source. The midicolumn was cut and placed in a 1.5
ml microcentrifuge tube. The tube was microcentrifuged at top speed for 2 minutes to remove any residual Column Wash Solution. Three hundred microliters of preheated TE buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA) was added to the midicolumn for 1 min. The DNA was eluted by centrifuging the midicolumn at top speed in a Eppendorf microcentrifuge for 2 min. The plasmid-DNA was stored in a sterile 1.5 ml microcentrifuge tube at -20°C for future use.

b) PCR Reactions

Primer Design

Template cDNAs for *fad2* and *fad6* were obtained from the Arabidopsis Biological Resource Centre. The cDNA sequences for *fad2* and *fad6* were obtained from GenBank using their GenBank accession numbers. Primers which were complementary to the first and last fifty base pairs of the full length cDNA sequences of the *fad* genes were designed using a DNASIS Sequence Analysis Software program. Forward and reverse oligomers of 18 bases were selected, with 50% GC contents. Based on the results obtained from the computer program, primers with the highest melting temperatures were selected for. Custom primers were made by Gibco BRL to be used for subsequent PCR reactions.

PCR

All PCR reactions were carried out in a Stratagene Robo-Cycler. All reactions were carried out in 50 µl volumes containing the appropriate buffer, 2
μg of each forward and reverse primer, 0.4 mM of each dNTP, 1 μg of plasmid template, and two units of Vent polymerase. All reactions were layered with two volumes of sterile mineral oil. The PCR reaction consisted of an initial denaturing step for 2 minutes at 94°C. This was followed by 30 cycles of the following conditions: 30 second denaturing step at 94°C, 60 second annealing of the primer at 57°C, and a 90 second extension of the primer at 72°C. The reaction products were stored at 4°C until further use.

**Restriction Endonuclease Digestion**

Genomic and cloned plasmid DNA (5 μg per reaction) were digested with a variety of restriction enzymes as per the manufacturer’s instructions. Digestions were performed in 1.5 ml microcentrifuge tubes at 37°C for a minimum of 2 hours, in reaction volumes of 25 to 50 μl. For cases of double enzyme digestions, reaction times were increased to overnight. In cases where simultaneous digestions were not possible, the enzyme requiring lower salt conditions was used first. The second endonuclease was added after conditions for digestion were optimized with the appropriate buffer.

**DNA Gel Electrophoresis**

DNA samples were electrophoresed on 0.7% agarose gels with 1X TAE (0.04 M Tris Acetate, 0.001 M EDTA, pH 8.0) as the running buffer. Agarose gels were stained in 0.5 μg/ml ethidium bromide (EtBr) and visualized on an
ultraviolet transilluminator (Fotodyne). Fragment sizes were determined by comparing DNA bands to those of *HindIII* digested λDNA.

**Low Melting Agarose**

DNA to be used as probes obtained from *E. coli* transformations and PCR reactions were run on 0.8 % low melting agarose gels, with 1 X TAE buffer containing 0.2 μg/ml EtBr. Low melting agarose gels were visualized on an ultraviolet transilluminator (Fotodyne). Probe sizes were determined through comparisons with *HindIII* digested λDNA molecular weight marker. Smallest possible bands were cut from the gels and transferred to sterile 1.5 ml eppendorf tubes. Samples were diluted with two volumes of dH₂O, and stored in 1.5 ml eppendorf tubes at -20°C until further use.

**RNA Gel Electrophoresis and Northern Blot Hybridization**

In order to ensure the equal loading of RNA samples for gel electrophoresis, 5 to 10 μg of total RNA was precipitated using 1/10 of total volume of 3.2 M sodium acetate and two volumes of absolute ethanol at -20°C for a minimum of 24 hours. RNA precipitates were spun out at full speed in the microcentrifuge at -4°C for 20 minutes. The ethanol supernatant was removed and RNA precipitates were dried for 2-3 minutes in a speed vacuum centrifuge. Fresh sample buffer was prepared each time, 3.5 μl of dH₂O-DEPC was added directly to the RNA pellets, and 16.8 μl of sample buffer was added. Samples
were placed at 65°C for 10 minutes and then on ice for three minutes. Three microliters of RNA loading buffer and 1 µl of ethidium bromide (10 µg/ml) were added to each sample. Samples containing between 5 and 10 µg of total RNA were separated on 1.1% (w/v) agarose gels containing formaldehyde (Sambrook et al., 1989). The gels were run on a Mupid system at 50 volts for approximately 45 minutes to one hour. The gels were then photographed in order to visually detect equal loadings of RNA per lane. The gels were then transferred to QIABRANE nylon membranes (Qiagen) using the capillary diffusion technique, using 20 x SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) as the transfer buffer (Sambrook et al., 1989). The transfer was permitted for at least 12 hours. The blots were then uv-crosslinked using a Stratagene cross linker at 1200 kJ for one minute. The blots were then baked for an additional 30 minutes at 80°C.

The membranes were then prehybridized for a minimum of 30 minutes at 65 °C using Amersham’s Rapid Hybridization tablets. Probes labelled with $^{32}$P were made using Pharmacia Biotech’s Quick Random Prime DNA labelling system kit. Procedures and conditions for labelling of the probes were as described by the instructions provided by the Quick Prime kit (Pharmacia Biotech).

DNA in low melting agarose was boiled for 3 minutes and placed at 37°C to cool. Thirty four microliters of denatured DNA was transferred to a sterile 1.5 ml eppendorf tube. Ten microliters of Rapid Random Primer mix was added to
the DNA, along with 5 μl $^{32}\text{P}$-dCTP and 1 μl of T7 polymerase. The DNA mixture was placed at 37°C for 15 minutes for labeling. Twenty-five microliters of stain (0.1 g Dextran blue, 0.1 g orange Na salt, 10 ml TE pH 8.0) was added to the mixture. The labelled probe was then eluted through a 1 ml syringe (plugged with glass wool), containing Sephadex G50. The volume of the blue eluent was measured and collected into a sterile 1.5 ml eppendorf tube. The radioactivity incorporated into the DNA probe was counted using a Beckman LS 6000 IC scintillation counter. The volume of probe required for a specific activity of 20 million cpm was determined and transferred to a sterile 1.5 ml eppendorf tube. The probe was heat denatured in a boiling water bath for 10 minutes and then placed on ice for 3 minutes. The probe was then added to the hybridization mixture.

Hybridization of the probes were carried out overnight at 65°C. Post-hybridization washes were carried out with decreasing SSC concentrations, using 2 x SSC with 0.1% (w/v) SDS (twice for 15 minutes); 0.5 x SSC with 0.1% (w/v) SDS (twice for 15 minutes); 0.1 x SSC with 0.1% (w/v) SDS (15 minutes at room temperature); and 0.1 x SSC with 0.1% (w/v) SDS at 65°C (times varied as the membranes were monitored using the Geiger counter). The membranes were then visualized by autoradiography using Kodak X-Omat-AR film with Kodak X-Omatic regular intensifying screens at -70°C for 24 to 96 hours.

The membranes were stripped by pouring boiling 0.1% (w/v) SDS solution with 2 mM EDTA (to prevent cleavage of the RNA) over the membranes. The membranes were agitated for a few minutes and a second wash was performed.
using fresh 0.1% (w/v) SDS solution with 2 mM EDTA with agitation. In order to ensure the efficient removal of the probe, the membranes were autoradiographed as outlined above.
RESULTS

Probe Preparation

Attempts were made to amplify the plasmids carrying the inserts (to be used as probes for northern blot hybridizations) through *E. coli* transformations. The plasmids carrying the *fad7/8* inserts were amplified using this method. For unknown reasons this method of amplification was unsuccessful for some of the plasmids. As a result, the inserts were amplified using PCR.

Primer Design and PCR Reactions

The use of the *fad2* and *fad6* DNA sequences obtained from GENBANK enabled the construction of custom primers (to be used for PCR), which were synthesized by GIBCO BRL Protogene. The sequences and melting temperatures of the forward and reverse primers used for PCR are listed in table 1. The table reveals the results obtained from the DNASIS sequence analysis program.

Low Melting Agarose Gel

The products from the PCR reactions were run on a 0.7% low melting agarose gel. The products obtained from the low melting agarose gel to be used as probes for subsequent labeling reactions for northern blot analysis are illustrated in figure 5. A *Hind*III λDNA molecular marker was run in order to
Table 1. Oligomers obtained using DNASIS sequence analysis program. Table shows the GC contents, melting temperatures, and forward and reverse sequences, of the oligomers. These oligomer sequences were constructed by Gibco BRL Protogene, for subsequent use in PCR reactions as primers, as described in the materials and methods.

<table>
<thead>
<tr>
<th></th>
<th>GC Content</th>
<th>Melting Temp.</th>
<th>Sequence</th>
<th>Forward/Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fad2</em></td>
<td>50%</td>
<td>59.3°C</td>
<td>TCGTAGGGTGTTCATCGT</td>
<td>forward</td>
</tr>
<tr>
<td><em>fad2</em></td>
<td>50%</td>
<td>57.2°C</td>
<td>CACACCTTTCTTGTCACC</td>
<td>reverse</td>
</tr>
<tr>
<td><em>fad6</em></td>
<td>50%</td>
<td>58.7°C</td>
<td>GGTCGCTTTCTTCGATT</td>
<td>forward</td>
</tr>
<tr>
<td><em>fad6</em></td>
<td>50%</td>
<td>56.7°C</td>
<td>GCGTTGGTGAAAGTGTTAG</td>
<td>reverse</td>
</tr>
</tbody>
</table>
Figure 5. PCR reaction mixture products were run on a 0.7% low melting agarose gel stained with EtBr to visualize amplified sequences. A 5µL sample of each reaction mixture was run to verify the band of interest alongside 15µL of the same sample.
provide an estimate of the lengths of the PCR products. A 5 μl sample of each reaction mixture was run alongside the PCR products, in order to verify the bands of interest. The results revealed that the *fad2* and *fad6* cDNA probes obtained were approximately 1.5-kb and 1.2-kb in length respectively.

**Regulation of Cytosolic Desaturase Enzyme Gene Expression**

**a) Effect of High Temperature on *fad2* Gene Expression**

Biochemical characterization of wild type *B. napus* plants grown at 20°C and shifted to 30°C suggested that the desaturase activity encoded for by the ω-6 cytosolic desaturase gene, resulted in a lower rate of desaturation of 18:1 fatty acids to 18:2 fatty acids (Williams *et al.*, 1995). To determine whether transcriptional regulation might play a role in the high temperature reduced expression of *fad2* activity in *B. napus* plants, the level of steady state *fad2* mRNA was measured in samples from *B. napus* subjected to different growth temperatures.

*B. napus* (Reston) plants were grown at 20°C for 3 weeks and then shifted to 30°C. Total RNA was extracted from 0 to 7 days and the photograph obtained from an ethidium bromide stained agarose gel containing 7.5 μg of total RNA loaded per lane is shown in figure 6 A. The results obtained from figure 6 A show that a relatively consistent amount of total RNA was loaded in each lane of the agarose gel. The northern blot analysis of *fad2* mRNA transcript accumulation in response to high temperature is revealed in figure 6 B. The *fad2*
Figure 6 A. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-6 cytosolic gene transcript accumulation during high and low temperature exposure (plants were grown at 20°C and shifted to 30°C from 0 to 7 days; 20-30, and plants were grown at 20°C and shifted to 5°C from 0 to 21 days; 20-5). Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
DNA probe hybridized to a band of about 1.2-kb in the RNA samples from wild type plants. *fad2* mRNA was visually detectable in all samples extracted. From days 0 to 3 there seemed to be little or no difference in *fad2* mRNA transcript levels. At days 4 and 5 there seemed to be a slight decrease in the *fad2* mRNA transcript accumulation when compared to days 0 to 3. At day 6 there seemed to be a slight increase in *fad2* transcript levels, and by day 7 the *fad2* transcript levels were equivalent to those of days 0 to 4. The results observed in figure 6 B, revealed no indication of a high temperature reduced expression of *fad2*, over the seven day period.

In a second experiment, *B. napus* plants (Reston) were grown at 20°C for 3 weeks, then acclimated to 5°C for 4 weeks, and then finally shifted to 30°C. Total RNA was extracted from 0 to 120 hours. Seven point five micrograms of total RNA was loaded per lane and the agarose gel was stained with ethidium bromide in order to visually compare the amount of total RNA per lane (figure 7 A). A northern blot (as shown in figure 7 B) was probed with a *fad2* A. *thaliana* cDNA. The *fad2* probe again hybridized to a band of about 1.2-kb in the RNA samples extracted. *fad2* mRNA was detectable in all samples extracted. At 0.5 hours there seemed to be a decrease in the *fad2* mRNA transcript level, when compared to times 0 and 0.25 hours. From 0.75 hours to 4 hours there seemed to be a gradual increase in the *fad2* mRNA levels, with the highest at 4 hours. From 4 to 6 hours the *fad2* mRNA transcript levels seemed to be relatively consistent. After 24 hours the level of *fad2* mRNA decreased. The differences in *fad2* gene expression observed visually in figure 7 B were not significant,
Figure 7 A. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-6 cytosolic gene transcript accumulation during low and high temperature exposure (plants were grown at 20°C for 3 weeks, shifted to 5°C for 4 weeks, and then shifted to 30°C). Total RNA was extracted at 30°C from 0 to 120 hours. Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
when the total RNA loaded (as shown in figure 7 A) per lane was taken into account. The results suggest that temperature had little or no affect on the levels of fad2 gene expression.

**Differences Between Plants Grown at 5°C and 20°C, and Shifted to 30°C**

In order to determine whether growth temperature had any effect on fad2 mRNA transcript accumulation in plants transferred to high temperature, figures 6 B and 7 B were visually compared. In summary, the results suggest that plants which were grown at 20°C, acclimated at 5°C, and then transferred to 30°C (figure 7 B) showed little change in fad2 transcript levels, as did plants which were grown at 20°C and shifted to 30°C (figure 7 B).

**b) Effect of Low Temperature on fad2 Gene Expression**

*B. napus* Plants

Williams et al. (1995), found that the ω-6 cytosolic desaturase activity was induced by growth at low temperature (Williams et al., 1995). To determine whether transcriptional regulation might play a role in the low temperature induced expression of ω-6 cytosolic desaturase activity, the steady state levels of fad2 mRNA were measured in samples from *B. napus* leaves.

*B. napus* plants (Reston) were grown at 20°C for 3 weeks and then shifted to 5°C. Total RNA was extracted at 5°C, from 0 to 21 days. Seven point five micrograms of total RNA was loaded per lane. The agarose gel was stained with
ethidium bromide to visually compare the amount of total RNA loaded per lane, as shown in figure 6 A. A northern blot (as shown in figure 6 B) was probed with a *fad*2 cDNA from *A. thaliana*. The *fad*2 probe hybridized to a band of about 1.2-kb. *fad*2 mRNA was detected in all samples extracted. These results indicated that there was an increase in *fad*2 mRNA transcript accumulation between 0 and 7 days, where after 7 days the transcript levels did not seem to change significantly. The increase in *fad*2 mRNA transcript between 0 and 7 days in figure 6 B, was not a significant increase when the total RNA loaded per lane was taken into account (see figure 6 A). As a result, there seemed to be no significant effect of low temperature on *fad*2 gene expression.

**HL29 and HL35 Plants**

Biochemical characterization of leaf lipids from a homozygous canola breeding line GS-272 of *B. napus* (HL29; wild type), resulted in the discovery of a temperature sensitive mutation (HL35) which affected the levels of unsaturation in chloroplast lipids (Williams *et al.*, 1997). Plants expressing this mutation exhibited lower levels of unsaturated fatty acids in all of the chloroplast lipids when plants were grown at low temperatures (5°C) but not at moderate (20°C) to high temperatures (30°C).

To determine whether transcriptional regulation might play a role in the low temperature reduced expression of ω-6 cytosolic desaturase activity in *B. napus* plants, the level of steady state *fad*2 mRNA was measured in plants subjected to different growth temperatures.
Double haploid *B. napus* plants (HL29 and HL35) were grown at 20°C and at 5°C. Total RNA was extracted and 7.5 µg of total RNA was run on an agarose gel, which was stained with ethidium bromide (figure 8 A). A northern blot was probed with a *fad2* cDNA from *A. thaliana* (figure 8 B). *fad2* mRNA was detected in all samples extracted. The results indicate that there was no significant difference in the *fad2* mRNA transcript accumulation between HL29 and HL35 plants grown at 5°C, and with HL35 plants grown at 5°C and 20°C. This suggests that *fad2* mRNA transcript accumulation was not affected by growth at low temperature, nor due to the presence of the mutation.

**Regulation of Chloroplastic Desaturase Enzyme Gene Expression**

**a) Effect of High Temperature on *fad6* Gene Expression**

Distribution of radioactivity in the major fatty acids of MGDG in the leaves of *B. napus* plants has shown that with increasing growth temperatures, the levels of radioactivity accumulating in the unsaturated fatty acids of MGDG decrease significantly (Williams *et al.*, 1995). Williams and Khan found that with increasing growth temperature, there was a significant decrease in ω-6 chloroplastic and ω-3 chloroplastic desaturase activity (Williams *et al.*, 1995).

To determine whether transcriptional regulation might play a role in the high temperature reduced expression of ω-6 chloroplastic desaturase activity in *B. napus* plants, the level of steady state *fad6* mRNA was measured in samples from *B. napus* (Reston) plants subjected to shifts in growth temperatures.
Figure 8 A. Ethidium bromide staining of 7.5 µg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-6 cytosolic gene transcript accumulation during low and high temperature exposure (plants were grown at 5°C, and at 20°C). Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
B. napus plants were grown at 20°C for 3 weeks and then shifted to 30°C. Total RNA was extracted from 1 to 7 days. Ten micrograms of total RNA was loaded per lane, and the agarose gel was stained with ethidium bromide (figure 9 A). The northern blot analysis of fad6 transcript accumulation in response to high temperature is illustrated in figure 9 B. The fad6 probe hybridized to a band of about 1.5-kb in the RNA samples from wild type plants. fad6 mRNA was detectable in all samples extracted. The results indicate that fad6 mRNA levels were highest at day 1, by day 2 there was a significant decrease in transcript levels. At days 3 and 4 fad6 mRNA levels increased, thereafter (days 5, 6 and 7) the transcript levels successively declined.

The northern blot from figure 7 B containing total RNA extracted from Reston leaves shifted from low to high temperature was stripped and reprobed with a fad6 A. thaliana cDNA, is shown in figure 10 B. The results reveal that the highest levels of fad6 mRNA transcript levels are observed between 0 and 1 hour. From 2 to 6 hours there is a significant decrease in the levels of fad6 mRNA transcripts. At 24 hours there seems to be a slight increase in transcript levels (in comparison to 6 hours), after which the levels of fad6 mRNA declines and remains constant. These results suggest that fad6 mRNA transcript levels tend to decline over time, in plants subjected to changes in growth from low to high temperatures.
Figure 9 A. Ethidium bromide staining of 10 μg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-6 chloroplastic gene transcript accumulation during high temperature exposure (plants were grown at 20°C, and then shifted to 30°C for seven days). Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
Figure 10 A. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-6 chloroplastic gene transcript accumulation during low and high temperature exposure (plants were grown at 20°C for 3 weeks, shifted to 5°C for 4 weeks, and then shifted to 30°C). Total RNA was extracted at 30°C from 0 to 120 hours. Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential blot hybridizations, as described in materials and methods.
Differences Between Plants Grown at 5°C and 20°C, and Shifted to 30°C

In order to determine whether growth temperature had any effect on \textit{fad6} mRNA transcript accumulation in plants transferred to high temperature, figures 10 B and 9 B were visually compared. In summary, the results reveal that plants which were grown at 20°C, acclimated at 5°C, and then transferred to 30°C (figure 10 B) showed a significant decrease in \textit{fad6} transcript levels, whereas plants which were grown at 20°C and shifted to 30°C (figure 9 B) showed little or no difference.

\textbf{b) Effect of Low Temperature on \textit{fad6} Gene Expression}

In order to determine whether transcriptional regulation might play a role in the low temperature reduced expression of \(\omega-6\) chloroplastic desaturase activity in HL29 and HL35 plants, the northern blot from figure 8 B was stripped and reprobed with a \textit{fad6} cDNA from \textit{A. thaliana} (figure 11 B). This experiment was also conducted to determine if the mutation in the HL35 plants affected the expression of the \textit{fad6} gene. The results from the experiments indicate that the temperature sensitive mutant (HL35) grown at 5°C accumulated significantly greater levels of \textit{fad6} mRNA transcript than HL35 plants grown at 20°C. The total RNA loaded from the HL29 plants grown at 5°C is less than the total RNA loaded from the HL35 plants grown at 5°C (figure 11 A). By using figure 11 A as a control for the amount of total RNA loaded per lane, the data indicate that there was no significant difference in \textit{fad6} mRNA transcript accumulation between the mutant (HL35) and wild type (HL29) plants grown at 5°C.
Figure 11 A. Ethidium bromide staining of 7.5 μg of total RNA loaded per lane. B. RNA gel-blot analysis of α-6 chloroplastic gene transcript accumulation during low and high temperature exposure (plants were grown at 5°C, and at 20°C). Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
Comparison of fad2 and fad6 Gene Expression in HL29 and HL35 Plants

In summary, the results indicate that HL35 plants grown at 5°C show a significant increase in fad6 gene expression when compared to HL35 plants grown at 20°C. Whereas, in the same plants, there is no detectable change in the expression of the fad2 gene at 5°C or 20°C, in both HL29 and HL35 plants.

Regulation of fad7/8 Gene Expression

a) Effect of High Temperature on fad7/8 Gene Expression

The northern blot from figure 9 B was stripped and reprobed with a fad7/8 cDNA (figure 12 B). The fad7/8 probe hybridized to a band of about 1.5-kb in the RNA samples from wild type (Reston) plants. fad7/8 mRNA was detectable in all samples extracted. The results indicate that the fad7/8 mRNA transcript accumulation was higher at day 4 in comparison to all of the other samples tested. At day 5 the transcript levels declined, by day 6 there seemed to be a further decrease, however at day 7 the transcript levels increased almost to those observed at day 4. From the analysis of figure 12 B, the results obtained indicate that plants subjected to high temperatures do not show any decrease in fad7/8 gene expression.
Figure 12 A. Ethidium bromide staining of 10 μg of total RNA loaded per lane. B. RNA gel-blot analysis of ω-3 chloroplastic gene transcript accumulation during high temperature exposure (plants were grown at 20°C and then shifted to 30°C for seven days). Total RNAs were separated using a denaturing agarose gel and transferred to a nylon membrane for sequential Northern blot hybridizations, as described in materials and methods.
DISCUSSION

Characterization of membrane bound desaturases by traditional biochemical approaches has been hindered by the problems of solubilizing and purifying membrane-bound proteins. Procedures involved in solubilizing integral membrane bound proteins have led to losses in desaturase activity. The increased understanding of the mechanisms and regulation of these desaturases has been obtained by the biochemical and genetic analysis of A. thaliana mutants, each one deficient in a desaturation step (Shah et al., 1997; Browse et al., 1988; Okuley et al., 1994; Miquel and Browse, 1992; McConn et al., 1994). Almost all of the fatty acid mutants of A. thaliana isolated so far have been shown to block a specific step in fatty acid or glycerolipid synthesis, resulting in reduced levels of the reaction’s products and accumulation of intermediates (Somerville and Browse, 1991).

fad6 versus fad2 Gene Expression

Based on the results obtained in this thesis work, the effects of temperature change on B. napus plants were more significant on the expression of the fad6 gene as compared to the fad2 gene. In addition, the expression of the fad6 gene was more significantly affected when plants were subjected to growth at 20°C, acclimated at 5°C, and shifted to 30°C, in comparison to plants grown at 20°C and shifted to 30°C. Furthermore, these results may suggest that
chloroplastic and cytosolic desaturase genes are regulated through different mechanisms in response to temperature stress.

When a plant is subjected to elevations in temperature, plants respond by decreasing their levels of unsaturated fatty acids (Williams et al., 1992). Therefore, it follows, that if the desaturation of fatty acids by desaturase enzymes is under genetic control, then at high temperatures there is a reduced requirement for desaturation events to occur (membranes are more fluid at high temperatures). This in turn should result in a decrease in desaturase gene expression. The fact that this result was observed in the expression of the \textit{fad6} gene, and not in the \textit{fad2} gene, reinforces the idea that the mechanisms involved in the regulation of chloroplastic and cytosolic desaturase enzyme gene expression in response to temperature stress may differ.

\textbf{fad2 Gene Expression}

In \textit{A. thaliana}, the \(\omega-6\) cytosolic desaturase is encoded by a single \textit{fad2} gene. Previous work on this gene has shown that \textit{fad2} is not regulated by low growth temperature (Okuley et al., 1994). Similarly, in soybean plants, Heppard \textit{et al.} (1996) found that the expression of the \textit{fad2} gene controlling fatty acid desaturation was not induced or enhanced by cold temperature. Likewise, from the results obtained from this thesis, no significant change in \textit{fad2} mRNA transcript accumulation could be detected at low temperatures in \textit{B. napus}.

On the other hand, at elevated growth temperatures, the transcript level of the \textit{fad2} gene in soybean plants has been observed to be higher than in plants
grown at lower temperatures (Heppard et al., 1996). Cheesbrough (1989) suggested that more transcript for ω-6 desaturase was synthesized to meet the needs of desaturation of fatty acids at higher temperatures, because ω-6 desaturase was found to be highly unstable at higher temperatures (Cheesbrough, 1989). These findings indicated that the increased levels of polyunsaturated fatty acids of soybean seed lipids by cold temperatures were likely the result of translational or posttranslational regulation, such as altered desaturase enzyme activity (Cheesbrough, 1989), rather than transcriptionally induced or enhanced expression of ω-6 desaturase genes in soybean plants. In contrast, the results from this thesis have shown that there is no detectable difference in fad2 mRNA transcript accumulation in B. napus plants subjected to high temperature. These results may suggest that a different mechanism is involved in regulating fad2 gene expression in B. napus plants, in comparison to soybean plants at high temperatures.

**fad6 Gene Expression**

A mutant of *A. thaliana* which lacked activity of the chloroplast glycerolipid ω-6 desaturase (fad6) contained reduced levels of 18-carbon and 16-carbon polyunsaturated fatty acids and increased levels of 18-1 and cis-16-1 precursors (Browse et al., 1988). Further genetic studies on fad6 revealed, that there were no significant differences in fad6 mRNA accumulation in mutant or wild type *A. thaliana*. The presence of normal levels of fad6 mRNA in leaves of mutant
plants indicated that the mutation which led to the deficiency in chloroplastic ω-6 desaturase activity did not influence the levels of fad6 mRNA transcripts. However, this did not rule out the possibility of transcriptional controls acting under other conditions or of other modes of regulation acting at the posttranscriptional level that could play a role in modulating fad6 gene expression (Falcone et al., 1994).

Vigh et al. (1993) demonstrated that decreases in growth temperature caused marked increases in transcript levels of the desA gene in cyanobacteria (Synechocystis PCC6803). In order to identify the mechanisms operating in the regulation of the desA gene, the degree of unsaturation of cyanobacterial membranes was experimentally decreased by catalytic hydrogenation, which as a result reduced membrane fluidity. This resulted in the enhanced expression of the desA gene in cyanobacterial cells. As a consequence of this result, Vigh et al. (1993) postulated that the primary signal in the perception of a change in temperature was a change in the fluidity of the plasma membrane, which in turn transduced the information to a mechanism which controlled gene expression (Vigh et al., 1993; Murata and Los, 1997; Falcone et al., 1994). If a similar system were present in higher plants to control the expression of the fad6 gene, the results obtained by Falcone et al. (1994) would have revealed increased transcript levels of fad6 mRNA in the mutant line of A. thaliana compared to wild type (Falcone et al., 1994). Since there were no differences noted in the expression of the fad6 gene in mutant and wild type A. thaliana indicates that a comparable mechanism is not present for fad6 gene expression.
Much of the previous research on fad6 has focused on the effects of low temperature on gene expression. The results from the experiments conducted in this thesis, revealed that plants acclimated to low temperatures and shifted to high temperatures, showed a significant decrease in fad6 mRNA transcript accumulation. These results, along with the previous work done by Falcone et al. (1994), may suggest that in higher plants there exist different mechanisms which control fad6 gene expression at low and high temperatures.

**fad7/8 Gene Expression**

The fad7 mutant of A. thaliana has been characterized as being deficient in the activity of chloroplast ω-3 fatty acid desaturase, which is responsible for the production of 16:3 and 18:3 fatty acids in the prokaryotic pathway of lipid synthesis. When A. thaliana plants were grown at low temperatures (10°C to 18°C), the quantity of trienoic fatty acids in leaves showed no difference between mutant or wild type plants. However at high temperatures (26°C), the trienoic fatty acid content decreased more significantly in the mutant in comparison to the trienoic fatty acid content in wild type plants (McConn et al., 1994). The observation that two independently isolated fad7 mutants displayed the same temperature-sensitive fatty acid profile suggested the existence of an additional cold induced ω-3 chloroplast desaturase, which may partially compensate for the defect in the fad7 mutant (Gibson et al., 1994; McConn et al., 1994). Evidence
for an additional desaturase was determined through the characterization of a mutation at a new locus designated as fad8 (McConn et al., 1994).

Genetic analysis of fad7 in wild type A. thaliana plants revealed that growth temperature had no effect on steady-state levels of fad7 mRNA. This suggested that, for this desaturase, transcriptional regulation by growth temperature was not an important factor in the cold acclimation response (Iba et al., 1993). However, plants may have other isozymes of some desaturases that are responsive to temperature. For example, the steady state level of fad8 (an isozyme of fad7) mRNA increased significantly in A. thaliana plants grown at 20°C as compared to 30°C (Gibson et al., 1994). This suggested that the differences observed in steady-state RNA levels could be the result of alterations in either the rate of fad8 transcription or in the stability of the fad8 mRNA.

The results reported here in this study indicate that temperature did not result in a decrease in the steady state levels of fad7/8 mRNA. The northern blots revealed increases in fad7/8 mRNA transcript levels at days 4 and 7, in comparison to the other days. These results are opposite to what would be expected in a shift from a moderate temperature (20°C) to a high temperature (30°C). It is already accepted that membrane fluidity is related to desaturation of fatty acids. At high temperatures the need for increased membrane fluidity should be reduced, thereby suggesting a decrease in desaturase activity. These results indicate that fad7/8 gene expression does not correlate with ω-3 chloroplastic desaturase activity at high temperature. This suggests that the
expression of the fad7/8 gene is not affected by exposure of B. napus to high temperatures.

**Desaturase Enzyme Activity**

Williams *et al.* (1988) have shown, that at low growth temperatures, *B. napus* plants displayed an increase in the levels of desaturase activity for both MGDG and PC (Williams *et al.*, 1988). The increase in desaturase activity, termed LTD, was shown to be a result of low temperature development as opposed to an immediate response to a change in environmental temperature. The desaturation rate of newly formed fatty acids in both MGDG (chloroplastic) and PC (cytosolic) was higher in plants grown at 5°C than in plants grown at 30°C. Through the use of $^{14}$CO$_2$ labeling of fatty acids and a computer simulation program (Williams *et al.*, 1993) the rates of desaturation of fatty acids in MGDG from plants grown at 5°C, 10°C, 20°C, and 30°C, and in plants grown at 20°C and 30°C after transfer to 5°C, were determined (Williams *et al.*, 1996).

Results from these experiments revealed a gradual increase over time in ω-9 and ω-6 desaturase activity, with plants subjected to decreases in growth temperatures. In contrast, ω-3 desaturase activity was less affected by growth temperature, which suggested that the primary control of desaturation rates lay within the ω-9 and ω-6 desaturases. *B. napus* plants which were initially grown at 20°C and 30°C and then transferred to 5°C, showed an increase in the level of ω-9 and ω-6 desaturase activity in C16, and of ω-6 desaturase activity in C18
fatty acids over 14 days (Williams et al., 1996). Williams et al. (1992) have postulated that the low rates of ω-9, ω-6, and ω-3 desaturase activities observed in plants at 30°C reflects a consistent level of unsaturation (BLD) of all membrane lipids (Williams et al., 1992). On the other hand, the rates of desaturation determined in plants grown at low temperatures were postulated to be due to an increase in a second process at low temperature (LTD). These findings suggest desaturase enzyme activity is correlated with different temperatures.

In this study it was shown that the steady state levels of fad6 transcript accumulation decreased only slightly in B. napus plants which were subjected to high temperature (30°C) over a 7 day period. These results indicate that there may be only a minor correlation between ω-6 chloroplastic desaturase enzyme activity and desaturase enzyme gene expression in B. napus plants subjected to high temperatures. On the other hand, there was no detectable change in the expression of the fad2 gene.

**HL29 and HL35**

Previous work by Williams et al. (1997) resulted in the discovery of a temperature sensitive mutant (HL35). This mutant exhibited lower levels of unsaturated fatty acids in all of the chloroplast lipids only in plants which were grown at 5°C.
In this study, the results clearly demonstrated that the expression of \textit{fad6} mRNA was induced at low temperature in HL35 plants. This result is not consistent with the idea of a correlation between desaturase activity and desaturase enzyme gene expression. If the activity correlated with gene expression in this case, then the expected results would have shown a similar or decreased level of \textit{fad6} transcript accumulation at low temperature. The fact that this was not observed indicates that the mutation affecting \(\omega 6\) chloroplastic desaturase activity in this mutant is presumably not due to the transcription of the \textit{fad6} gene itself. Rather, there is some other mechanism affecting \(\omega 6\) desaturase activity at low temperatures. Studies in mutants of \textit{A. thaliana} indicated that the mutation which led to the deficiency in chloroplastic \(\omega 6\) desaturase activity did not influence the accumulation of the \textit{fad6} transcripts (Falcone et al., 1994).

These results, however, do not rule out the possibility that, although the \textit{fad6} transcript is expressed, it may be unstable and may not be processed into a functional enzyme. Another possibility is that, if the mutation is in the \textit{fad6} gene then more of the enzyme is required at low temperatures to overcome its deficiencies caused by the mutation. Finally, the relative abundance of \textit{fad6} mRNA transcripts between wild type and mutant plants grown at 5°C do not appear to differ significantly. Since the \textit{fad6} transcript accumulation in the mutant behaves like the wild type plant at low temperature, this then strengthens the possibility that the mutation causing the lower levels of all chloroplast lipids at
low temperature in the mutant is not related to the expression of the \textit{fad6} gene itself.

\textbf{Conclusion}

The objective of this research was to gain a better understanding into the role of temperature and its effects on desaturase enzyme gene expression (by observing mRNA transcript levels), and to provide insight into correlations between desaturation enzyme activity and gene expression. Although the findings from the work of this thesis are subtle, the general conclusions are:

1. Plants grown at 20\(^\circ\)C and shifted to 30\(^\circ\)C showed only a slight decrease in \textit{fad6} mRNA transcript accumulation, whereas plants grown at 20\(^\circ\)C, acclimated at 5\(^\circ\)C, and shifted to 30\(^\circ\)C showed a significant decrease in \textit{fad6} transcript accumulation. Furthermore, HL35 plants grown at 5\(^\circ\)C showed a significant increase in \textit{fad6} mRNA transcripts in comparison to plants grown at 20\(^\circ\)C. This is consistent with the idea of a low temperature stimulation of desaturase activity (from 20\(^\circ\)C to 5\(^\circ\)C) and high temperature (30\(^\circ\)C) thermal lability of desaturase enzymes. This may indicate that there may be a correlation between \textit{fad6} gene expression with \(\omega\)-6 chloroplastic desaturase enzyme activity.

2. There were no detectable changes in the expression patterns of the \textit{fad2} genes at any of the temperatures tested, suggesting little or no correlation between \textit{fad2} mRNA transcript levels and \(\omega\)-6 cytosolic desaturase activity.
at any temperature.

3. The results obtained indicate that chloroplastic desaturase enzyme gene expression is more affected by changes in temperature as compared to the expression of cytosolic desaturase enzymes. This indicated that, chloroplastic and cytosolic desaturase enzymes may be subject to different mechanisms that regulate the expression of their respective genes in response to changes in temperature.

**Future Studies**

The experiments in this thesis were designed to determine the effects of temperature on desaturase enzyme gene expression in *B. napus* plants. The work presented primarily focused on the use of Northern blots in assessing desaturase enzyme gene expression. Other experiments of interest would be to determine the signal transduction pathways involved in temperature stress responses in *B. napus* plants. Also with the use of *in vivo* footprinting techniques, one could determine which sites on desaturase enzyme genes are occupied during transcription. With the use of site directed mutagenesis, mutations could be introduced to occupied DNA sequences upstream and downstream of the transcription start site. This might elucidate the regulatory sequences involved in the transcription of desaturase genes.

Also through the extraction and purification of *trans*-acting factors bound to *cis*-acting DNA regulatory sequences, one could identify key regulatory elements involved in the transcription of desaturase genes with respect to
temperature stress. Other experiments of interest would be to look at heat shock proteins or cold inducible genes with respect to their roles in temperature stress in relation to desaturase activity, to determine any correlation.
References


selectivities of glycerol-3-phosphate acyltransferase and
monoacylglycerol-3-phosphate acyltransferase from pea and spinach

ture-regulated gene encoding a chloroplast ω-3 desaturase from Arabidopsis

Gray, G., Chauvin, L., Sarhan, F., Huner, N. (1997) Cold acclimation and

Harwood, J.L. (1996) Review: Recent advances in the biosynthesis of plant fatty

The Biochemistry of Plants, Stumpf, P.K., Conn, E.E., eds., Academic

growth temperature regulation of two different microsomal ω-6

Cloning of a higher-plant plastid ω-6 fatty acid desaturase cDNA and its

of photosynthesis and altered chloroplast ultrastructure in a mutant of

Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V., Hugly, S.,
Somerville, C. (1993) A gene encoding a chloroplast ω-3 fatty acid
desaturase complements alterations in fatty acid desaturation and
chloroplast copy number of the fad7 mutant Arabidopsis Thaliana. The
J. Biol. Chem. 268: 24099-24105.

Imperial, V. (1998) Molecular species analysis of the glycerolipids involved in
fatty acid desaturation in Brassica napus, M.Sc. thesis.

Johnson, G. and Williams, J. (1989) Effect of growth temperature on the
biosynthesis of chloroplastic galactosyldiacylglycerol molecular species


