A MOLECULAR GENETIC APPROACH TO REDUCING THE SATURATED FATTY ACID CONTENT OF CANOLA OIL

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ABSTRACT

Brassica napus is known to contain an endogenous and soluble stearoyl-acyl carrier protein ($^{\Delta 9}$18:0-ACP) desaturase, but does not express a palmitic (16:0)-ACP desaturase. Levels of 16:0 are low in canola oil and are associated with enhanced cholesterol biosynthesis in humans. In an attempt to further reduce the saturated fatty acid (SFA) content of canola oil, *B. napus* L. cv Westar was transformed with a cDNA encoding a $^{\Delta 9}$16:0-ACP desaturase from cat's claw (*Doxantha unguis-cati* L.). *Arabidopsis thaliana* was also transformed with this cDNA. Transformation of both oilseeds resulted in increased production of palmitoleic acid ($^{\Delta 9}$16:1) and many other effects on fatty acid composition. Overall, the SFA content did not decrease in either oilseed and investigation to why this effect occurred was examined using transgenic *B. napus*. Molecular genetic testing on second generation *B. napus* also determined the plants contained the cDNA of interest and were transcribing the cDNA.

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8:0	caprylic acid/caprylate
10:0	capric acid/caprate
12:0	lauric acid/laurate
14:0	myristic acid/myristate
16:0	palmitic acid/palmitate
$^{\Delta 9}$16:1	palmitoleic acid/palmitoleate
18:0	stearic acid/sterate
$^{\Delta 6}$18:1	petroselinic acid/petrosenlinate
$^{\Delta 9}$18:1	oleic acid/oleate
$^{\Delta 11}$18:1	vaccenic acid/vaccenate
$^{\Delta 9,12}$18:2	linoleic acid/linoleate
$^{\Delta 6,9,12}$18:3	γ-linolenic acid/linolenate
$^{\Delta 9,12,15}$18:3	α-linolenic acid/linoleate
$^{\Delta 6,9,12,15}$ 18:4	octadecatetraenoic
$^{\Delta 11}$20:1	eicosenoic acid/eicosenoate
$^{\Delta 5,8,11,14}$20:4	arachidonic acid
$^{\Delta 13}$22:1	eurcic acid/eucate
$^{\Delta 4,7,10,13,16,19}$22:6	docosahexanoic acid
ACBP	acyl-CoA binding protein
ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein

ACS	acyl-CoA synthetase
ANOVA	analysis of variance
ARC	Alberta Research Council
AT	acyltransferase
A. thaliana	*Arabidopsis thaliana*
BC	biotin carboxylase
B. juncea	*Brassica juncea*
Black current	*Ribes nigrum*
B. napus	*Brassica napus*
Borage	*Borago officinalis*
bp	base pairs
B. rapa	*Brassica rapa*
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CDP-CPT	ctyidine diphosphocholine diacylglycerol choline phosphotransferase
Castor bean	*Ricinis communis* L
Cat's claw	*Doxantha unguis- cati*
CoA	coenzyme A
Coconut	*Cocos nucifera*
Coriander	*Coriander sativum*
Cotton	*Gossypium herbaceum*
Cucumber	*Cucumis sativus*
DAG	*sn*-1,2-diacylglycerol

DAP	days after pollination
DGAT	diacylglycerol acyltransferase
DGTA	diacylgylcerol transacylase
DPA	days post anthesis
dsRNA	double stranded RNA
EFA	essential fatty acids
EFTu	elongation factor Tu
EMS	ethyl methanesulfonate
ENR	enoyl-ACP reductase
ER	endoplastic reticulum
Evening Primrose	*Denothera biennis*
FA	fatty acid
FAMEs	fatty acid methyl esters
FAS	fatty acid synthesis
Fd	ferredoxin
FdR	ferredoxin oxireductase
G3P	*sn*-glycerol-3-phosphate
Geranium	*Pelargonium* x *hortorum*
GLC	gas liquid chromatography
GPAT	*sn*-glycerol-3-phosphate acyltransferase
GUS	β-glucuronidase
Hawk's beard	*Crepis palaestina*
HDL	high density lipoprotein

HIP	hexane isopropanol
hpRNA	hairpin RNA
IAA	isoamyl alcohol
KAS III	β-ketoacyl-ACP synthase III
KCS	3-ketoacyl-CoA synthase
KR	3-ketoacyl-ACP reductase
LDL	low density lipoprotein
LFAH12	bifunctional oleate 12-hydroxylase promoter from *Lesquerella fendleri*
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
LR-NMR	low resolution nuclear magnetic resonance
MAG	monoacylglycerol
MCFAs	medium chain fatty acids
Meadowfoam	*Limnanthes alba alba*
MHCl	methanolic HCl
Milkweed	*Asclepias syriaca*
MS	Muashigi and Skoog
MUFA	monounsaturated fatty acids
NaMeth	Sodium methoxide
NptII	neomycin phosphotransferase
Oil palm	*Elaies guineensis*
Olive	*Olea europea*

PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
Parsley	*Petroselinum crispum*
PC	phosphatidylcholine
PDAT	phospholipid:diacylglycerol acyltransferase
PE	petroleum ether
Peanut	*Arachis hypogaea* L.
PG	phosphatidylglycerol
PL	phospholipase
PLD	polar lipid
PS	photosystem
PTGS	post transcriptional gene silencing
PUFA	polyunsaturated fatty acids
rbcS-E9	rubisco small unit polyadenlyation terminator
RT-PCR	reverse transcription-polymerase chain reaction
Safflower	*Carthamus tinctorius*
SDM	site directed mutagenesis
Sesame	*Sesamum indicum* L.
SFA	saturated fatty acid
-SGPAT	non-transformed GPAT spinach containing plants
sn	steriochemical numbering
Soybean	*Glycine max*
Spinach	*Spinacia oleracea*

Squash	*Cucurbita pepo*
Sunflower	*Helianthus annuus*
TAG	triacylglycerol
T-DNA	transferred DNA
TE	thioesterase
TLC	thin layer chromatography
Tobacco	*Nicotiana tabacum*
Tomato	*Lycopersicon esculentum*
USFA	unsaturated fatty acids
White spruce	*Picea glauca*
WS	*A. thaliana* ecotype
WT	wild type
Yeast	*Saccharomyces cerevisiae*

1.0 Introduction

Genetic engineering is an effective means of producing canola oil with desired fatty acid (FA) composition (Moon et *al.* 2000). Health conscious customers can benefit from seeds oils with low levels of saturated fatty acid (SFA). In order to maintain status as a "low saturation oil" the SFA level present in canola oil must remain below 7%. The maintenance of a low level of saturation allows for the canola oil market to compete with the low SFA soybean oils being developed in the U.S. by companies such as Dupont and Pioneer Hi-Bred. In recent years the level of SFAs in canola oil have increased from 6%, ten years ago, to above 7% in 1998. This increase is a direct result of a change in the variety of canola grown, from *Brassica rapa* (Polish type) to *B. napus* (Argentine type), because *B. napus* generates higher yields. In an attempt to maintain the low levels of saturation in *B. napus*, a desaturase gene from *Doxantha unguis-cati L.* (cat's claw) was inserted via *Agrobacterium* mediated plant transformation and was expressed using a seed specific promoter from *Lesquerella fendleri*. Cat's claw desaturase catalyzes the conversion of palmitate (16:0)-acyl carrier protein (ACP) to produce palmitoleate ($^{\Delta 9}$16:1)-ACP. *B. napus* does not contain a 16:0-ACP desaturase endogenously, and as a result retains approximately 5% 16:0 incorporated into its seed oil. The goal of this project was to decrease the amount of 16:0, through conversion to $^{\Delta 9}$16:1, thereby attempting to increase the overall unsaturated fatty acid (USFA) content of *B. napus* oil.

Two canola lines, coded C_2-31A and C_2-41, were used in the investigation because they had the highest $^{\Delta 9}$16:1/16:0 ratio (0.11 and 0.10, respectively) of the transformants examined. These plant lines and also untransformed Westar controls were grown in a greenhouse during the summer and fall of 2001. Molecular genetic testing

was performed on the plant lines to determine if the plants were transgenic for the cDNA of cat's claw 16:0-ACP desaturase and also the transcription of that cDNA within the *B. napus* genome. The FA profile from mature seeds was investigated to examine the effects of the transformation with the cDNA encoding 16:0-ACP cat's claw desaturase. Also, the acyl-CoA pool at 28 days post anthesis (DPA) was investigated to determine if there were higher amounts of $^{\Delta 9}$16:1 and vaccenate ($^{\Delta 11}$18:1), the elongation product of $^{\Delta 9}$16:1, present in the acyl-CoA pool compared with the FAs in the mature seeds.

Preliminary experiments, using *Arabidopsis thaliana* transformed with a *35S* promoter, were performed prior to the transformation of the *B. napus*. For *Arabidopsis thaliana* the total acyl FAs were extracted from the leaves and seeds and triacylglycerol (TAG) from the seed total lipid was also extracted, and tested to determine the effect of the ubiquitous expression of cat's claw desaturase on the FA profile of transformed *A. thaliana*.

2.0 LITERATURE REVIEW

2.1 Economic Importance and Nutritional Value of Canola Oil

On a global scale vegetable oil production accounts for about 50 million tons annually (Voelker and Kinney 2001). Canola (*B. napus and B. rapa,* respectively) or low eurcic acid ($^{\Delta 13}$22:1) rapeseed was developed through plant breeding effects because $^{\Delta 13}$22:1 was declared to be potentially harmful and caused smooth muscle lesions (Knauf and Facciotti 1995). Now, canola is guaranteed to contain less than 2% $^{\Delta 13}$22:1, and is also known to be low in glucosinolates (Daun 1986, Dupont et al. 1989). Canola was developed by the introduction of recessive alleles at two loci that control the synthesis of $^{\Delta 13}$22:1. $^{\Delta 13}$22:1 is synthesized by the successive elongation of oleic acid ($^{\Delta 9}$18:1), thus it is not surprising that mutations at these two loci result in the formation of canola oil with the most predominant FA being $^{\Delta 9}$18:1 (Puyaubert et al. 2001). Canola is devoid of monounsaturated FAs longer than 22 carbons (Puyaubert et al. 2001), with $^{\Delta 9}$18:1 accounting for ~60% of the FA composition (Perry 1993b). In Canada alone, canola contributes five billion dollars to the Canadian farmgate and is a major contributor to Canada's net trade balance (personal communication Dave Wilkins from Canola Council of Canada, Jan. 2003). The use of canola oil by people is diverse and includes food and industrial applications. The oil is known for its health benefits derived from the FA composition.

Due to the increase in cardiovascular and coronary heart disease, an optimal diet low in SFAs and high in USFAs has been recommended (Feldman 1999). In Canada and the U.S., coronary heart disease is a major health concern and accounts for 50 % of

premature deaths. SFA consumption is associated with elevated blood concentrations of total and low-density lipoprotein (LDL) cholesterols, whereas USFAs are associated with high density lipoprotein (HDL) cholesterol and lower incidences of both cardiovascular and coronary heart disease (Feldman 1999).

USFAs include both monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). MUFAs contain one point of unsaturation, whereas PUFAs contain more than one point of unsaturation. Although MUFAs are not considered as neutral FAs they are known to lower total and LDL cholesterol levels without lowering levels of HDL cholesterol (Feldman 1999, Kris-Etherton et al. 1999, McDonald et al. 1989). PUFAs, like MUFAs, are known to enhance immune function but with greater effects (Pond 1998). PUFAs include essential fatty acids (EFAs), FAs that the human body cannot make, such as α-linolenic acid ($^{\Delta 9,12,15}$18:3) and linoleic acid ($^{\Delta 9,12}$18:2). These FAs are used as precursors to synthesize docosahexaenoic acid ($^{\Delta 4,7,10,13,16,19}$22:6) and arachidonic acid ($^{\Delta 5,8,11,14}$20:4), respectively, and are enriched in the central nervous system (Carlson 2000). Plant oils are often a source of USFAs, and as a result provide benefits when consumed (Lands 1997).

Canola oil has the lowest SFA content of any other commonly consumed vegetable oil on the market (Figure 1, Canola Council of Canada). Canola oil does not contain lauric (12:0) and myristic (14:0) SFAs, and the levels of 16:0 are the lowest of any vegetable or animal fat (Johnson et al. 2002b, Canola Council of Canada). Although these SFAs are cholesterol-raising FAs, Lindsey et al. (1990) found replacing 16:0 for both 12:0 and 14:0 in the diet caused an increase in HDL cholesterol suggesting that chain length had an effect on health. Canola oil also contains stearic acid (18:0), which

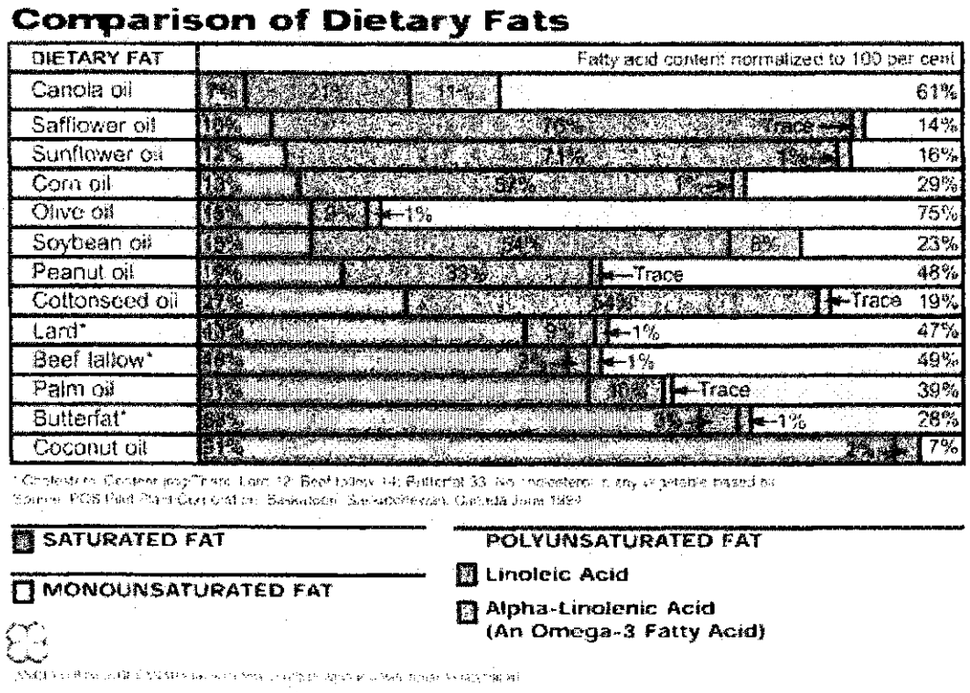

Figure 1. Comparison of dietary fats and oils. The fatty acid content, including saturated, monounsaturated, and polyunsaturated FAs, of commonly consumed dietary fats. All FA contents are normalized to 100 %. Taken from the Canola Council of Canada (http://www.canola-council.org/).

does not seem to affect blood cholesterol level (Lindsey et al. 1990), a moderate level of $^{\Delta 9,12}$18:2 (21%), and appreciable amounts of $^{\Delta 9,12,15}$18:3 (11%). The major FA present in canola oil is $^{\Delta 9}$18:1 accounting for about 61 % of the total FA content. As stated earlier, $^{\Delta 9}$18:1 FA is known to lower total and LDL cholesterols levels without lowering levels of HDL cholesterol (Feldman 1999, Kris-Etherton et al. 1999, McDonald et al. 1989). Overall, the low SFA content and beneficial MUFAs and PUFAs make canola oil a healthy choice as a dietary fat source.

2.2 FA and TAG Biosynthesis in Developing Oilseeds

2.2.1 Structure and Function of TAG

The structure of TAG can be seen in Figure 2, where three FAs are esterifed to the glycerol backbone via the hydroxyl groups. The glycerol backbone has three distinct stereochemical numbering (*sn*) positions 1, 2, and 3, due to its lack of rotational symmetry (Weselake 2002). Oilseeds have undergone stereochemical identification, where SFAs are usually found in the *sn*-1, USFAs are usually found in the *sn*-2 position and variable FAs are found in *sn*-3 positions (Wilberg et al. 1997). Here, the use of specific phospholipases (PLs) enable a FA to be removed from the glycerol backbone in any of the three positions (Willams et al. 1995).

Seed TAG represents a carbon source that provides energy to enable germination (Voelker and Kinney 2001). The energy in oilseeds is usually in the storage form of TAG and accumulates in the embryo and or the endosperm during seed maturation and undergoes catabolism to ensure early seedling development. The TAG molecules are stored in oil bodies that are coated by a single layer of phospholipid. Oleosins embedded

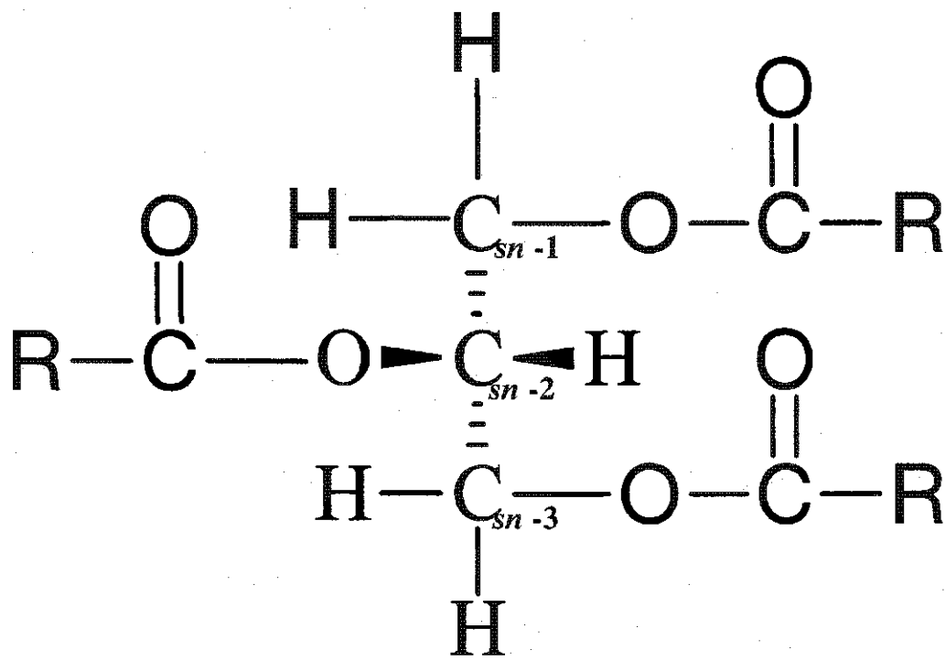

Figure 2. The Structure of TAG. The glycerol backbone is esterified via the hydroxyl ends of FAs, and contains three FAs in total. The three positions on the glycerol backbone are labeled as *sn*-1, 2, and 3 due to lack of rotational symmetry. The "R" groups are the fatty acid chains without the carboxyl group. In *B. napus* oilseeds the *sn*-1 and 3 positions tend to be occupied by SFAs whereas the *sn*-2 position tends to be associated by USFAs. Adapted from Weselake (2002).

in to the surface of oil bodies prevent droplets of oil from coalescing during desiccation of the seed (Lacey and Hills 1996).

2.2.2 FA Biosynthesis in the Plastid

The biosynthesis of oil in oilseeds starts from carbohydrate sources including glucose, fructose, and sucrose (Hill and Rawsthorne 2000). These metabolic products of photosynthesis yield the acetyl coenzyme A (CoA) substrate for *de novo* fatty acid synthesis (FAS) (Harwood 1996).

The *de novo* biosynthesis of FAs occurs in the stroma of the plastid, the prokaryotic pathway, where the stepwise condensation of 2C units produces acyl chains (See Figure 3) (Slabas et al. 2001b). The metabolic precursors of the synthase complex are produced from the ATP dependent reaction of acetyl-CoA and bicarbonate catalyzed by acetyl-CoA carboxylase (ACCase) to produce malonyl-CoA, making this the committed step in FA biosynthesis (Slabas et al. 2001a). The role of ACCase has been investigated by Post-Beittenmiller et al. (1992) who provided evidence of the regulatory role that ACCase plays in chloroplast FA biosynthesis. Next, malonyl-CoA is converted to malonyl-ACP via malonyl-CoA:ACP transacylase. The next four reactions are necessary for chain elongation and are also catalyzed by different enzymes. The first step in the acetyl chain elongation occurs with the condensation of acetyl-CoA and malonyl-ACP to form 3-ketobutyl-ACP and CO_2 via the β-ketoacyl-ACP synthase III (KAS III).

The ACP unit is a small, 9kD, acidic protein which acts as an arm to aid in the production of the FA elongation product. (Ohlrogge and Browse 1995). There are a

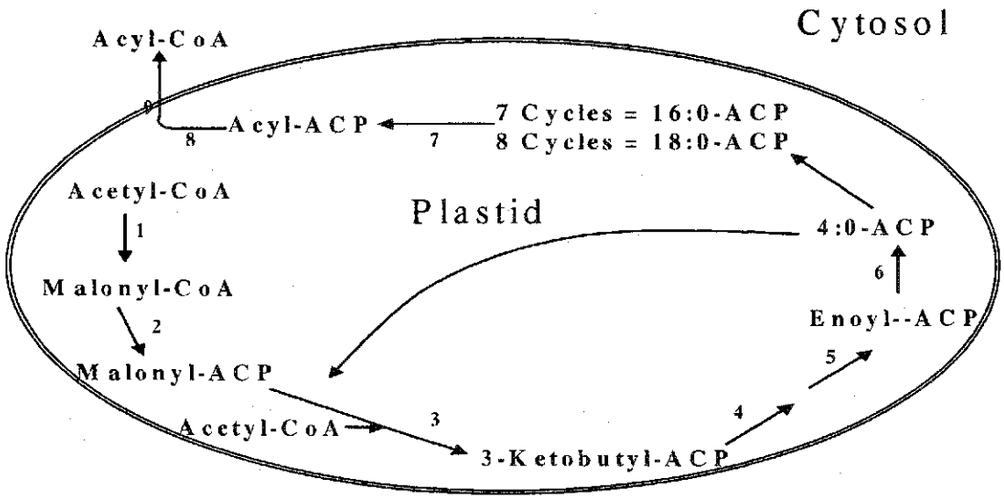

Figure 3. FA biosynthesis in plants. This occurs in the plastid where stepwise condensation of two carbon units produces acyl chains. The mechanism starts with acetyl-CoA, where ACCase (1) catalyzes the production of malonyl-CoA from acetyl-CoA (Johnson et al. 2002). Malonyl-CoA is then catalyzed by MCAT (2) to produce malonyl-ACP (Suh et al. 2002). The malonyl-ACP and acetyl-CoA undergo condensation via KAS III (3) (Slabas et al. 2001) to produce 3-ketobutyl-ACP. Next, reduction occurs via 3-ketobutylacyl-ACP reductase (4) (Johnson et al. 2000), followed by dehydration with 3-hydroxylacyl-ACP (5) (Wiberg et al. 2000). A last reduction occurs through catalytic action of enoyl-ACP reductase (6) to produce 4:0-ACP. This product is subsequently cycled around again with malonyl-ACP for further elongation until the FA is 16 or 18 carbons in length. The 18:0-ACP is then catalyzed by acyl-ACP TE (7) (Voelker and Kinney 2001) to move across the plastidial envelope. Prior to entering the cytosol, acyl-ACP is catalyzed by acyl-ACP hydrolyase (8) to remove the ACP unit, and then with CoA to produce acyl-CoA and is catalyzed by acyl-CoA synthetase (9) (Hill and Rawsthorne 2000). Adapted from Somerville et al. (2000).

number of isoforms of ACP and many of them have yet to be elucidated. Branen et al. (2001) over expressed *Arabidopsis thaliana* ACP-1, a major isoform present in the seed, using the Cauliflower Mosaic Virus 35S (CaMV35S) promoter. Western and Northern analysis showed there was a 3 - 8 fold increase in the expression of ACP-1 in leaf tissue, but there were no significant changes in expression levels in the seed. The increase in the ACP-1 isoform in the leaves also altered the FA composition in the leaves causing a decrease in 16:3 and an increase in 18:3, indicating that overexpression of an ACP isoform can change FA composition in plants (Branen et al. 2001).

Both KAS III and ACCase are important regulatory enzymes and can affect the flux in FAS (Roesler et al. 1997). This idea was further investigated by Verwoert et al. (1995), when the *Escherichia coil fabH* gene was isolated and the effect of the overproduction of this bacterial KAS III on *B. napus* was determined. Significant increases in KAS III activities were found in transgenic plants resulting in effects on the FA profile of the seed oil, although this did not cause a significant change in the total lipid biosynthetic flux. The changes in FA profile included a decrease in $^{\Delta 9}$18:1 and concomitant increase in $^{\Delta 9,12}$18:2 and $^{\Delta 9,12,15}$18:3. These results suggested that KAS III may exert some control over FAS. In a similar study by Dehesh et al. (2001), the overexpression of *Ch KAS III*-1 and *Ch KAS III*-2 genes from *Cuphea hookeriana* caused a significant decrease in the total FA and an increase in the amount of 16:0 present. The increase in KAS III also caused a lower expression of KAS II, and was suspected to be a result of less malonyl-ACP available to KAS II. As stated earlier, ACCase is thought to limit FAS. In an experiment by Roesler et al. (1997), the effect of inserting cytosolic *ACC1*, an *A. thaliana* ACCase gene, into *B. napus* was investigated using a napin

promoter fused to the *A. thaliana ACC1* to target the plastid. The insertion of *ACC1* into *B. napus* caused a 10 - 20 fold increase of ACCase in maturing seeds, and also an increase of 5% in the seed oil content. Here, the FA profile of the transformants showed an increase in $^{\Delta 9}$18:1 and eicosenoate ($^{\Delta 11}$20:1), and a decrease in $^{\Delta 9,12}$18:2, $^{\Delta 9,12,15}$18:3 and $^{\Delta 13}$22:1 content. If ACCase was thought to be the main enzyme in the regulation of FAS, then overexpression of KAS III should have had little effect on the flux, but in the examples above this was not the case. Nikolau et al. (2000) produced a series of *A. thaliana* plants that had reduced levels of acetyl-CoA metabolizing enzymes including ACCase, ATP citrate lyase, acetyl-CoA synthetase, and plastidial pyruvate dehydrogenase. To date the investigators have shown that reducing the ACCase expression by 20 - 30% of the wildtype (WT) caused a significant reduction in the growth of the plants. Evidence also indicated that ACCase has a substantial role in affecting the amount of FA that is synthesized. Turnham and Northcote (1983), found that *B. napus* lipids began to accumulate 16 days after pollination (DAP) and rapidly increased until lipid synthesis began to plateau at about 28 DAP, resulting in about 60 times the amount of lipid compared to the initial level. The ACCase activity also increased 14 DAP to peak at 24 DAP, rising 64 fold over that period with the highest rate at day 16, and then decreased rapidly.

Following condensation, reduction occurs to produce 3-hydroxylacyl-ACP via the catalytic action of 3-ketoacyl-ACP reductase (KR). Next, dehydration occurs to produce enoyl-ACP via the catalytic action of 3-hydroxylacyl-ACP dehydrase, and lastly a second reduction occurs to form the elongated 4:0-ACP product. The last reduction is catalyzed by enoyl-ACP reductase (ENR) (Ohlrogge and Jaworski 1997). The 4:0-ACP then

undergoes subsequent rounds of condensation reactions with malonyl-ACP until 16:0-ACP is synthesized. These subsequent reactions are catalyzed by the enzyme KAS I, except in the last reaction producing 18:0-ACP, where the reaction of 16:0-ACP and malonyl-ACP is catalyzed by KAS II. The FAs present in most oilseeds are 16C and 18C units in length. More than 90% of FAs produced in WT *B. napus* seeds have a chain length of 16C or longer (Eccleston and Ohlrogge 1998).

Some genes that regulate the FA biosynthesis have been investigated. O' Hara et al. (2002) investigated the mole ratios of the FA synthesizing enzymes over embryogenesis. The peak of mRNA accumulation of all the enzymes from developing *B. napus* seeds tested occurred between 20 to 29 DPA, with drastic reductions at 32 DPA. The steady state for all the enzymes occurred at 42 DPA, where all transcripts fell to about 5% of their peak levels, signifying that the mRNAs have similar stability and kinetics during synthesis. The proteins and enzymes tested included ACP, biotin carboxylase (BC), one of the four subunits that make up ACCase, KR, ENR, and thioesterase (TE). Previously, Ke et al. (2000) reported that the four components of chloroplastic ACCase maintain a constant molar ratio of mRNA in siliques throughout development. O' Hara et al. (2002) reported that the molar ratios of the enzymes were at a constant ratio throughout embryogenesis, but the absolute levels were different. The ratios of the transcripts were calculated to be 1.0:0.6:1.2:0.4:6.9 for KR:BC:ENR:TE:ACP, respectively. It was apparent that the transcript abundance of the mRNAs for each of the catalytic proteins (KR, BC, ENR, and TE) were relatively similar, whereas the number of transcripts for the ACP was 7 fold higher that that of KR. The regression coefficients for each of these data are greater than 0.88, clearly suggesting that

the mRNA levels involved in lipid biosynthesis accumulate at a constant ratio throughout embyrogenesis.

2.2.3 Desaturation of Acyl-ACP in the Plastid

Desaturation can occur at the level of the plastid in plants. In *B. napus*, the soluble $^{\Delta 9}$18:0-ACP desaturase acts upon 18:0-ACP to produce $^{\Delta 9}$18:1-ACP, and accounts for the major FA in the seed oil. The involvement of $^{\Delta 9}$18:0-ACP desaturase in the seed oil was clearly demonstrated by Knutzon et al. (1992) using antisense–RNA gene expression in both *B. napus* and *B. rapa*. Here, the decrease in $^{\Delta 9}$18:0-ACP desaturase activity caused a dramatic decrease in the oil content. The specificity towards 18:0-ACP substrates is over 100 fold greater than for 16:0-ACP (Harwood 1996). A number of other oilseeds also contain soluble acyl-ACP desaturases with low specificity for 16:0-ACP, including *Glycine max* (soybean), *Carthamus tinctorius* (safflower) and *Ricinis communis* L (castor bean) (Tocher et al. 1998, Cahoon et al. 1998). The expression of $^{\Delta 9}$18:0-ACP desaturase mRNA during seed development has been followed by Slocombe et al. (1992, 1994) in *B. napus*. The transcript was first detected at 25 DPA, peaked at 45 DPA and decreased thereafter.

One plant known to have a soluble ACP-desaturase with increased preference for 16:0-ACP is cat's claw, with 64% of the FA present in the seed oil as $^{\Delta 9}$16:1 (Chisholm and Hopkins 1964). The homology in the amino acid sequences between cat's claw $^{\Delta 9}$16:0-ACP desaturase and the $^{\Delta 9}$18:0-ACP desaturase from castor bean is 85% (Cahoon et al. 1998). To determine the substrate specificity, Cahoon et al. (1998) performed an

amino acid substitution of castor bean $^{\Delta 9}$18:0-ACP desaturase from leucine 118 to a tryptophan. This substitution resulted in an 80 fold increase in substrate specificity towards 16:0-ACP indicating that a small difference in amino acid sequence can dramatically change the desaturase preference towards a FA. The homology of many amino acid sequences from desaturases have been investigated by Harwood (1996). The precursor polypeptides (with a signal peptide attached) of *Spinacia oleracea* (spinach) 18:0-ACP desaturase shares 84, 81, and 82% homology, respectively, to the precursor polypeptides from castor bean, *Cucumis sativus* (cucumber) and safflower.

Desaturation is an oxidation reaction that occurs under aerobic conditions with the requirement of two electrons, and one molecule of oxygen (Bloomfield and Bloch 1958, Los and Murata 1998). All known acyl-ACP desaturase enzymes use ferredoxin (Fd) as the electron-donating cofactor (Schultz et al. 2000). There are two classes of Fds, including the photosynthetic and heterotrophic class. The photosynthetic Fds are light-regulated and expressed in photosynthetic tissue, whereas the heterotrophic Fds are independent of light and are found more ubiquitously (Schultz et al. 2000). Schultz et al. (2000) examined the influence of the different isoforms of Fd on acyl-ACP desaturases in *Coriander sativum* (coriander), *Thunbergia alata* and *Pelargonium* x *hortorum* (geranium). Here, the heterotrophic Fd isoform showed 20 fold higher activity with acyl-ACP desaturase in all three plants when compared to the photosynthetic Fd isoforms. The heteotrophic Fd also increased the ubiquitous $^{\Delta 9}$18:0-ACP desaturase 1.5 - 3.0 fold in both seed and leaf tissue. These results suggested that the Fd isoforms that specifically interact with acyl-ACP desaturases to achieve optimal enzyme activity are heterotrophic isoforms of Fd. It has been suggested the each acyl-ACP desaturase binds two atoms of

iron to form reactive complexes with oxygen, Fe-O-Fe (Los and Murata 1998). This complex removes two H atoms, one from each C, and the bond between the C molecules changes from a C-C single bond to a C=C double bond in the carbon chain of the FA substrate. See section 2.3 for more on desaturases.

2.2.4 Hydrolysis of Acyl-ACPs in the Plastid

Once the acyl chain is assembled and desaturation has occurred, termination of FA elongation occurs via the action of an acyl-ACP TE (Harwood 1996). Acyl-ACPs can also serve as substrates for plastidial acyltransferase (AT). For example, acyl-ACP: *sn*-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) catalyzes action of *sn*-glycerol-3-phosphate (G3P) to generate lysophosphatidic acid (LPA) (Murphy 1994, Facciotti et al. 1999). Different plants are known to contain TEs with specificities for various endogenous chain lengths. A comparison of over 30 plant TE sequences revealed there are two classes: FatA and FatB (Jones et al. 1995, Voelker and Kinney 2001). The FatA TEs act preferentially towards $^{\Delta 9}$18:1-ACP, whereas FatB TEs prefer saturated acyl-ACPs with lengths from 8C to 16C. The K_m for both 16:0 and $^{\Delta 9}$18:1 substrates were investigated by Hellyer et al. (1992) and were found to be almost identical, at about 1μM, implying the binding affinity of the enzyme for the substrate is not a function of the chain length, but rather the enzyme recognizes the ACP moiety for binding. The V_{max} for the $^{\Delta 9}$18:1 substrate was considerably higher than for 16:0, indicating that once the FA is bound, both length and degree of unsaturation determine the reaction velocity. The difference in the V_{max} also suggests why more $^{\Delta 9}$18:1 than 16:0 is exported from the

plastid. As an example, the TE in the California bay plant has a preference for 12:0-ACP. Eccleston and Ohlrogge (1998) studied the expression of this TE in developing seeds of transgenic *B. napus* and found that it resulted in up to 60 mole% of laurate in TAG. Although the total seed oil was not reduced, levels of β-oxidation also increased. This increase in β-oxidation was explained as a maintenance mechanism to dispose of excess FAs in order to prevent the interference of the excess FAs with other enzyme systems. Other changes included higher levels of ACP and acyl-ACP, particularly medium chain length variants. Several other enzymes of FAS were increased 2 – 3 fold at the mid stage of development including ACCase, 18:1-ACP desaturase and KAS III. This upregulation of enzymes enabled the transgenic plants to maintain the same amount of total FAs as was present in the WT plants. In a similar study, Wiberg et al. (2000) found that developing and mature transgenic *B. napus* seeds accumulated up to 7 mole% of caprylate (8:0), 29 mol % caprate (10:0) or 63 mole% of 12:0. The accumulation of 8:0 and 10:0 resulted from the overexpression of the medium chain TE, *Ch* FatB2, from California bay in *B. napus* when crossed with a line overexpressing *Cocos nucifera* (coconut) lysophosphatidic acid acyltransferase (EC 2.3.1.51) (LPAAT), an enzyme responsible for the acylation of LPA to generate phosphatidate.

2.2.5 FA Export from the Plastid and Biosynthesis of Acyl-CoAs

After termination of FA elongation in the chloroplast, acyl-ACP is converted to free fatty acids via acyl-ACP hydrolase, and then to their CoA thiol-ester derivatives by the catalytic action of acyl-CoA synthetase (ACS) in the outer plastidial envelope

(Johnson et al. 2002a). Johnson et al. determined the rate of acyl chain export from the plastid in *B. napus*. The investigators separated the *in vitro* synthesis of the FAs from export by making use of acyl-CoA binding protein (ACBP), which are 10 kDa ubiquitous proteins found in eukaryotes that specifically bind to long chain acyl-CoAs. After the FAs were synthesized, CoA was added to the plastids and their acyl chains were converted to acyl-CoAs and exported into the incubation medium. The export rate was dependent on the acyl-CoA concentation. The medium containing the highest rate of export contained acyl-CoA concentrations in the range of 10-20 μM, whereas one-half the maximum export rate could be attained at a concentration of 2.5 μM. The group also found that the maximal rate of acyl chain export was comparable to the rate of FA synthesis before export was initiated, at 110 nmol acyl chains U^{-1} GAPdH h^{-1} (glyceraldehyde-3-phosphate dehydrogenase). The concentration of ACBP has been estimated by Fox et al. (2000) to be between 10-30 μM and is somewhat greater than that of the general concentration of the acyl CoA pool. The exact mechanism of export of acyl-CoAs is not known, but it is hypothesized that the acyl-CoAs leave the plastid bound to ACBPs. A cDNA encoding an ACBP homologue has been cloned from *B.* napus (Hills et al. 1994).

2.2.6 FA Elongation in the Endoplasmic Reticulum (ER)

Once FAs are built and after ACS catalyzes the formation of acyl-CoA TEs, FA elongation can occur further in the ER. ACS was investigated by Domergue et al. (1999) and Hlousel-Radojcic et al. (1998), and does not directly play a role in FA elongation.

Rather, the genes named *Bn-FAE1.1* and *Bn-FAE1.2* control 3-ketoacyl-CoA synthase (KCS) and are responsible for FA elongation. Puyaubert et al. (2001) examined the difference in the expression of these two genes in cultivars of *B. napus*. The first was Gaspard, a rapeseed containing high $^{\Delta 13}$22:1, and the second was ISLR4, a low $^{\Delta 13}$22:1 rapeseed. Although the *Bn-FAE1.1* gene expression was similar in both cultivars, *Bn-FAE1.1* was expressed at a higher level than that of *Bn-FAE1.2*. The difference between these two cultivars resulted in the identification of the protein KCS encoded by the gene *Bn-FAE1.1* and was determined by using anti-FAE1 antibodies prepared against a recombinant Bn-FAE1.1 fusion protein. This resulted in no protein detection for KCS at any developmental stage of ISLR4. The absence or limitation of KCS is in agreement with others because low $^{\Delta 13}$22:1 cultivars of canola are known to have mutations in KCS genes that lead to lower levels of $^{\Delta 13}$22:1 (Han et al. 2001, Slabas et al. 2001a).

2.2.7 TAG Biosynthesis

Acyl-CoAs produced through the catalytic action of ACS and elongases become part of an acyl-CoA pool located in the cytosol. Here the acyl-CoA pool can be used as a substrate for the ATs of phospholipid and TAG biosynthesis located in the ER. The TAG biosynthesis pathway is known as the Kennedy pathway (Johnson et al. 2000) (Figure 4). The pathway uses acyl-CoAs as acyl donors and G3P as the initial acceptor. The glycerol backbone undergoes sequential acylations via AT action. The AT catalyzes the acyl portion of the acyl-CoA to be added to the *sn*-1 position is GPAT, and is known to draw upon both saturated and unsaturated FAs to yield 1–acylglycerol-3-phosphate or LPA

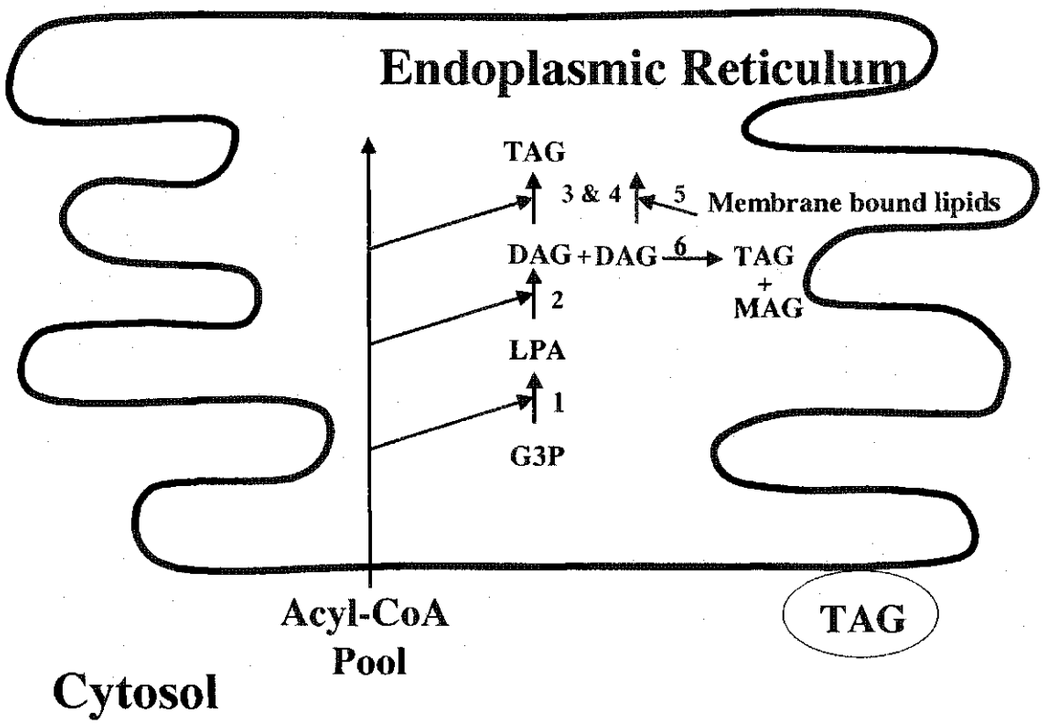

Figure 4. TAG biosynthesis. In the ER, FAs from acyl-CoA are added to produce LPA catalyzed by GPAT (1) (Johnson et al. 2002). Next, another acyl group from the acyl-CoA pool is added to produce DAG via the catalytic action of LPAAT (2). The removal of phosphate from PA is catalyzed by PAP (Slabas et al. 2001) (3), and the final acyl-CoA dependent acylation is catalyzed by DGAT (4). Another mechanism for producing TAG can occur with DAG accepting a FA from PC catalyzed by PDAT (5) (Wiberg et al. 2000). Lastly, DGTA (6) can catalyze the transfer of a FA between two DAG molecules to produce TAG and MAG (Stobart et al. 1997). Adapted from Somerville et al. (2000).

(Murata and Tasaka 1997). There are three types of GPAT; with the different isoforms present in the chloroplast, mitochondria and cytoplasm. The type being referred to here is the cytoplasmic GPAT, as this form is a hydrophobic protein that is bound to the ER (Murata and Tasaka 1997). The next AT to catalyze the *sn*-2 position is LPAAT yielding phosphatidic acid (PA) (Slabas et al. 2001b). In oilseeds, LPAAT appears to be the most acyl-specific ATs among the three-glycerol ATs (Sun et al. 1988), and does not use SFAs including 12:0 and 16:0. The enzyme is also known to exclude 22:1 from this position (Sun et al. 1988, Slabas et al. 2001a). Although LPAAT does not use SFAs for substrates, Knutzon et al. (1999) demonstrated that *B. napus* could be genetically engineered to incorporate 12:0 at the *sn*-2 position. The *B. napus* line was produced by crossing *B. napus* transformed with California bay cDNA encoding 12:0-ACP TE with *B. napus* transformed with coconut cDNA encoding a 12:0-CoA-preferring LPAAT. Some plants of this cross had up to 75% 12:0 at the *sn*-2 position. Another group of investigators transformed high $^{\Delta 13}$22:1 *B. napus* with an AT from *Limnanthes alba alba* (meadowfoam) (Lassner et al. 1995). Although this transformation did not change the amount of $^{\Delta 13}$22:1 present in the total lipid, it did increase the amount of $^{\Delta 13}$22:1 found at the *sn*-2 position and resulted in a concomitant decrease in $^{\Delta 13}$22:1 in *sn*-1 and *sn*-3 positions. Prior to acylation at the *sn*-3 position, a phosphate group must be removed from PA by phosphatidic acid phosphatase (PAP) (EC 3.1.3.4) (Stobart et al. 1997). After the phosphate group is removed from the glycerol backbone, the third and final AT, diacylglycerol acyltransferase (DGAT) (EC 2.3.1.20), catalyzes the addition of the last acyl group to form TAG (Weselake 2002). DGAT activity is also thought to limit the flow of carbon, resulting in a high flux control coefficient in TAG formation (Weselake

et al. 1993, Perry 1993b, Ichihara et al. 1988, Perry et al. 1999). In a study by Weselake et al. (1993), DGAT activity was found to be maximal during the active phase of lipid accumulation in *B. napus* seeds where DGAT activity increased from 14 DPA to a maximum level at 33 DPA and then decreased until 40 DPA.

Some plants are known to have acyl-CoA independent mechanisms for TAG production. Examples of these include both phospholipid: diacylglycerol acyltransferase (PDAT) (E.C. 2.3.1.158) and diacylgylcerol transacylase (DGTA). PDAT catalyzes the transfer of FA from a phosphatidylcholine (PC) donor to a *sn*-1,2-diacylglycerol (DAG) molecule, the acceptor. PDAT appears to play a major role in removing unusual FAs from phospholipids in *Helianthus annuus* (sunflower), castor bean and *Crepis palaestina* (hawk's beard) (Banas et al. 2000). Here, the unusual FAs can be removed from the phospholipids and incorporated into TAG, where most unusual FAs are found (see section 2.3.8 for more detail about unusual FAs). Dahlqvist et al. (2000) found that the specificity of PDAT was different for varying plant species. Ricinoleoyl and vernoloyl groups of PC were incorporated into TAG from castor bean, and hawk's beard, respectively. Another example of acyl-CoA independent reactions producing TAG involves DGTA. Two DAG molecules react through the catalytic action of DGTA to produce TAG and monoacylglycerol (MAG) (Stobart et al. 1997), and the enzyme was first identified in rat intestine by Lehner and Kurksis (1993).

Although major glycerolipids are first synthesized using the FAs 16:0 and $^{\Delta 9}$18:1, subsequent desaturation reactions of these FAs can occur by membrane bound desaturases in the chloroplast and ER (Ohlrogge and Browse 1995). The advancement in understanding the regulation and mechanism of these desaturases occurred by the

characterization of seven *A. thaliana* mutants. Here, the loci that control each of these are *fad* genes. Mutations of different *fad* genes control desaturation at different locations. For example, the mutation at the *fad2* and *fad3* loci affects desaturation of the extrachloroplast lipids, whereas mutations in *fad4* through to *fad8* affect chloroplast desaturation. See Tocher et al. (1998) and Los and Murata (1998) for extensive reviews on desaturases and also section 2.3 of this thesis.

2.2.8 Incorporation of Unusual FA into TAG

Many plants contain unusual FAs in their seed storage lipids that occur naturally or as a result of genetic engineering. These unusual FAs vary in chain length, degree of unsaturation, position of unsaturation or can contain functional groups such as hydroxy, epoxy, cyclic, and acetylenic groups (Roscoe et al. 2002). Plants can tolerate large amounts of unusual FAs in the storage lipids because they are sequestered into oil bodies (Millar et al. 2000). Wiberg et al. (1997) investigated the distribution of two transgenic lines of 12:0 producing *B. napus*. Both medium and high 12:0 producing lines were investigated. The medium and high lines accumulated 34 mole% and 55 mole% of 12:0 in TAG fraction, respectively, whereas the PC fraction was only composed of 2.7 mole% and 6.6 mole% of 12:0 at seed maturity, respectively. Although the majority of unusual FAs eventually end up in TAG molecules, these unusual FAs are often a part of PC first. To determine the role that PC plays in TAG formation Schultz and Ohlrogge (2000) have examined the seed oil of *T. alata*. *T. alata* contains an endogenous $^{\Delta 6}$16:0-ACP desaturase resulting in oil with petroselinic acid ($^{\Delta 6}$18:1) accounting for 80% of the TAG composition, with most of this coming from the PC fraction. Eccleston et al. (1996) have

also looked into the regulation of unusual FAs in plants. Here, the investigators expressed the *35S-FatB1* gene into *B. napus*. The transgenic plants accumulated high levels of medium chain fatty acids (MCFAs) into their TAG without detection of the MCFAs anywhere else in the plant, even though higher levels of *FatB1* were present in vegetative tissues other than the seed. The investigators proposed that the MCFAs were being synthesized in the leaves and then broken down because the enzymes from both the β-oxidation and the glyoxylate cycles were induced in the leaves of the *35S-FatB1* transgenic *B. napus* plants (Eccleston and Ohlrogge 1998).

Two theories have been proposed to explain the targeting of the unusual FAs to storage lipids and their exclusion from membrane lipids. These theories include specificity of enzymes and compartmentalization (Roscoe et al. 2002). Enzymes having various specificities include TEs, ATs, ctyidine diphosphocholine diacylglycerol choline phosphotransferase (CDP-CPT), PLs and PDAT (Roscoe et al. 2002). Compartmentalization was proposed as an alternative to enzyme specificity whereby the membrane lipid and TAG assembly might be carried out in separate domains of the ER. A study on *B. napus* zygotic embryos by Lacey and Hills (1996) provided evidence for the difference in the localization of membrane lipid and TAG biosynthesis. The investigators prepared sub-cellular fractions using a sucrose gradient, and tested the fractions for enzyme activity. The high-density fraction closely matched the marker enzymes of the ER, including LPAAT and CDP-CPT. Analysis of the lipids extracted from the low-density fraction revealed that 50 mole% were from TAG, and were compared to only 15 mole% in the bulk ER. This suggested that the low-density

membranes are derived from the portion of the ER that is involved in the formation and secretion of TAG.

2.2.9 TAG Accumulation in Relation to Embryo Development in *B. napus*

The accumulation of lipid in *B. napus* follows a typical pattern for oil rich tissues (Perry and Harwood 1993a). There are three phases of oil development. These include the initial stage of rapid cell division where little lipid synthesis takes place as fertilization has just occurred. The next phase includes rapid accumulation of TAG, and the third and final phase has little TAG deposition because desiccation of the seed takes place. These three phases include 0-18, 18-40 and 40-65 DPA (mature), respectively. Changes in lipid content in developing *B. napus* cv. Shiralee were investigated by Perry and Harwood (1993a), where the rapid deposition of TAG began at 16 DPA and continued until about 40 DPA. This data was in agreement with number of important lipid-synthesizing enzymes that were mentioned in section 2.2.2, and have been shown to be more active during the second phase, 18-40 DPA (O' Hara et al. 2002). The classes of lipid investigated included TAG, DAG and polar lipids (PLD). At the earliest stage of development, TAG was the most abundant lipid class, representing 83.5% of the lipid content. DAG represented 6.7% and PLD 9.8% of the lipid content, with the majority of the lipid accounted for by PC. During seed development, the percentage of TAG continually increased until around 40 DPA where the steady state was 95%. Also, at maturity, the amount of DAG and PLD only accounted for 1.2 and 3.7%, respectively, with the PLD content similar to that in the mature embryo at 46 DPA. It is important to note that canola grown in a greenhouse matures faster when compared to canola grown in

a field setting and causes a reduction of about 20% in oil production as a longer maturation period usually increases oil yield (Perry and Harwood 1993a). Murphy and Cummins (1989) conducted a similar study, except the *B. napus* cv. Bienvenue was grown in a field setting. In this experiment the first stage occurred between 14 and 21 DPA (two to three weeks), while the rapid phase of TAG biosynthesis, stage two, occurred during the period 28-42 DPA (four to six weeks), where the production of TAG then leveled off until dehydration occurred. The plants were harvested at 133 DPA (12 weeks).

Investigators are also working to understand the factors that limit the flux of TAG produced. Bao and Ohlrogge (1999) found the supply of FAs limited the accumulation of TAG. This was further supported by Ramli et al. (2002) where flux biosynthesis of both FA synthesis (block A) and lipid assembly (block B) were investigated in *Elaies guineensis* (oil palm) and *Olea europea* (olive). Here, the biosynthesis flux coefficients where higher in both oil palm and olive for block A, suggesting that FA synthesis had more control in regulating the amount of TAG produced in the overall production of oil. Regardless of the mechanism controlling TAG production, the *B. napus* oil is composed of common FAs. In mature seeds, the TAG, DAG and PLD content are 95.4, 1.5, and 3.1%, respectively (Murphy and Cummins 1989), and the values are in agreement with the study by Perry and Harwood (1993a). The major FAs present in *B. napus* seed reported by both Perry and Harwood (1993a) and Murphy and Cummins (1989) were 16:0 (<5%), 18:0 (<2%), 18:1 (~60%), 18:2 (~20%), 18:3 (~10%) and others (<1%).

2.2.10. FA Composition of Acylglycerols in Relation to Chilling Tolerance

While an increase in USFAs present in membrane lipids helps to maintain membrane fluidity, Murata et al. (1982) discovered the correlation between the nature of the FAs in the *sn*-1 position of phosphatidylglycerol (PG) in the chloroplasts and the sensitivity of a particular plant species to chill tolerance. Chill resistance plants had 0 - 20% of 16:0 esterified to the *sn*-1 position while chill sensitive plants exhibited 25 - 65% 16:0 at this position. In a recent study, Ariizumi et al. (2002) reported an increase in the USFAs in PG from the leaves of transgenic rice when grown at low temperatures. Rice leaves were genetically modified to contain *cis* USFAs from 19.3% in the WT to 29.4 and 32.0% in T1 plants segregated with cDNAs encoding GPAT of the chloroplasts from *A. thaliana* and spinach, respectively, and to 21.4% in the non-transformed GPAT spinach containing plants (-SGPAT). While all plants had the same amount of $O_{2\,(g)}$ evolution at 25°C, the plants containing *A. thaliana* and spinach GPAT had less of a reduction of $O_{2\,(g)}$ evolution when placed at 5° and 11°C when compared to the WT and the -SGPAT plants. Also, the level of USFAs in the PG fraction of both the *A. thaliana* and spinach GPAT containing plants showed less impaired rates of $O_{2\,(g)}$ evolution from leaves when compared to the WT and the -SGPAT plants at 14 and 17°C. This result indicated that the photosynthetic rate was maintained in the transformed plant at lower temperatures.

Other research has been done to test the effect of low temperature photoinhibition on plants. Low temperature photoinhibition occurs when photosynthesis is impeded when plants are exposed to light at low temperature. Photoinhibition is known to target photosystem (PS) II where damage to the D1 protein impairs electron transport (Moon et

al. 1995). When the D1 protein at the PS II center is damaged renaturation of this protein involves several steps (Nishida 1996). The recovery of the D1 protein has been hypothesized to decrease in time as USFA content increases in membrane lipids. An example of this was performed by Moon et al. (1995), where tobacco transformed with a cDNA encoding squash GPAT resulted in plants with a decrease in the amount of *cis* USFAs in the PG fraction of thylakoid membranes. The purpose of this experiment was to determine the relationship of the USFA content in the thylakoid membrane lipids and the low temperature photoinhibition. The isolated thylakoid membranes in the transformed, null vector control and WT tobacco underwent the same photoinhibitory effect when placed at temperatures of 5, 15 and 25°C, indicating the USFA content of PG does not affect the photoinhibition of PS II activity in thylakoid membranes (Moon et al. 1995). Rather, the differences found were associated with an increase in recovery time of photosynthetic rate in the isolated membranes from the transgenic tobacco when kept at lower temperatures and then moved to 25°C when compared to the null vector control and WT tobacco. These results indicated that the USFAs of PG in the thylakoid membrane help to stabilize against photoinhibition of the photosynthetic machinery (Moon et al. 1995). A similar study by Vijayan & Browse (2002) showed mutants *fad1*, *fad5*, *fad6* and *fad 3-2fad7fad8* of *A. thaliana* had reduced USFA content in the thylakoid membranes when compared to WT. The three mutants *fad5*, *fad6* and *fad 3-2fad7fad8* were more susceptible to photoinhibition, whereas the *fad1* mutant behaved the same as the WT. The triple mutant, *fad 3-2fad7fad8*, while it had the same rate of photoinactivation of PS II as the WT and other mutant plants, did not contain any trienoic FAs and had longer recovery periods, relative to WT at temperatures lower than 27°C.

The results of Vijayan & Browse (2002) indicate that trienoic FAs of the thylakoid membrane also help to maintain low temperature recovery from photoinhibition in *A. thaliana*.

2.3 Properties of Desaturases

In plants there are three types of desaturases including ACP-desaturase and glycerolipid desaturase, and the just recently discovered acyl-CoA-like desaturases (Cahoon et al. 2000, Marilla et al. 2002). These desaturases catalyze the introduction of double bonds into FAs esterified to acyl-ACP, glycerolipids, or acyl-CoA, respectively. As stated earlier, desaturases can act in the plastid or ER. The acyl-ACP desaturases act at the plastidial level, whereas glycerolipid desaturases work at both the membranes of the plastid and ER level. The acyl-CoA desaturase is operative in the cytoplasm.

At any level, in order for desaturation to be successful, the reaction requires molecular oxygen to function (Los and Murata 1998, Bloomfield and Bloch 1958). Molecular oxygen is split and adds to two protons to produce two molecules of water (Sperling and Heinz 2001). Based on spectroscopy, X-ray crystallography and the amino acid sequence, it seems most likely that the desaturase contains a di-iron complex (Sperling and Heinz 2001). In the unreactive form, two iron ions of the complex are coupled by a bridging oxygen atom ligand that forms a μ-oxo-di-iron complex. After the reductive removal of the oxygen and replacement with molecular oxygen, the resulting μ - peroxo complex gains catalytic ability by rearranging and splitting the oxygen molecule to form the reactive bis-μ-oxo-di-ferryl (Fe^{IV}). This activated center then catalyzes the stepwise removal of two hydrogen substrates with their combination to one

of the oxygen atoms, leaving the second in the μ-oxo-di-iron complex and ready to start a new cycle.

An example of a desaturase with this mechanism is the $^{\Delta 9}$18:0-ACP desaturase (EC 1.14.99.6) containing a di-iron structure within a four helix bundle of the protein. The crystal structure of $^{\Delta 9}$18:0-ACP desaturase was deduced by overexpressing the cDNA in *E. coil* and making sufficient crystals (Lindqvist et al. 1996). X-ray crystallography studies revealed a tunnel in the protein lined with hydrophobic residues to accommodate the 18C substrate. When the 18C acyl chain is inserted into the tunnel a bend is caused in the middle of the acyl chain and the H on the ninth and tenth Cs are presented to the active site.

Another requirement for desaturation includes electron donors. For plastidial desaturases, both soluble and membrane bound, the source of the electron donor is Fd (Los and Murata 1998, Sperling and Heinz 2001). Under photosynthetic conditions, Fd is the electron donor in PS I, whereas in the dark or non-photosynthetic plastids the electrons are derived from the soluble flavoprotein ferredoxin:NADP oxidoreductase (FdR) (Sperling and Heinz 2001). In the case of glycerolipid desaturases in the ER, the electron donor is usually cytochrome b_5 (Los and Murata 1998). Cytochrome b_5 recieves electrons from NADH, or in part from NADH:cytochrome b_5 oxireductase or NADPH cytochrome P450 oxireductase (Sperling and Heinz 2001). The electron donor for the acyl-CoA-like desaturases in plants have not been identified.

In order for desaturase activity to occur, the metal center of the desaturase is kept in place by the ligand shells of the amino acids such as histidine, glutamine, glutamic acid and aspartic acid. The primary sequences cause substrate specificities that are

characteristic of desaturases (Sperling and Heinz 2001). Together, the amino acid residues are believed to contribute to metal chelation and the overall amino acid sequences for identification of the type of desaturase. With the exception of acyl-ACP desaturases, the desaturases, including glycerolipid and acyl-CoA desaturases, have three characteristic histidine boxes. Other enzymes that have histidine boxes include hydroxylases from castor bean, which also have sequence homology to desaturases (van de Loo et al. 1995).

The first level of desaturation occurs at the plastidial level in plants. After FA bioassembly occurs in the plastid, desaturase is known to act upon an acyl chain before the hydrolysis of the chain from the ACP. Although one of the major FAs exported from the plastid is $^{\Delta 9}$18:1, thus making the major desaturase in the plastid the Δ9 desaturase, other major FAs exported from the plastid include 16:0 and 18:0. There has been much work on Δ9 desaturases. Refer to section 2.2.3, for an overview of some of the research done to date. Other studies on Δ9 include the research on the activity in developing cotyledons of sunflower (Griffiths et al. 1998). The result of this investigation showed that developing cotyledons of sunflower at the most active stage of oil synthesis had stimulated Δ9 desaturase when NADPH, Fd, and FdR were added. The removal of endogenous Fd from the soluble enzyme by gel filtration showed complete loss of Δ9 desaturase activity, although activity was restored when exogenous Fd and FdR were provided to the reaction mixture. Lastly, when Fd was replaced with a soluble form of cytochrome b_5, lacking a hydrophobic anchoring domain, no desaturation resulted, proving further the specificity of the binding site is towards Fd. The Fd donor was further verified when Fd inhibitors such as bis (salicylidene)-1,3-propanediamine were

added and caused a reduction in the Δ9 desaturase activity. Due to the importance of the $^{\Delta 9}$18:0-ACP desaturase, the isolation of several cDNA clones have been made from species including soybean, jojoba, safflower, rapeseed, sunflower and oil plam (Griffiths et al. 1998, Shah et al. 2000). Although the idea that the first unit of unsaturation occurs at the plastidial level has generally been accepted, there is evidence that this may not be the case. Examples of these include homologs of the Δ9 acyl-glycerolipid desaturases (commonly found in cyanobacteria) which are not localized within the plastid, and have been cloned from rose petals (Fukuchi-Mizutani et al. 1995) and *A. thaliana* (Fukuchi-Mizutani et al. 1998).

While 18:0-ACP or Δ9 desaturase are thought to be the first substrate and desaturase, respectively, this is not always the case. An example of a substrate other than 18:0-ACP includes 16:0-ACP in the production of $^{\Delta 9}$16:1 in the seed oil of cat's claw (Chisholm and Hopkins 1964), and also in *Asclepias syriaca* (milkweed). The production of $^{\Delta 9}$16:1 is the result of the desaturation of 16:0-ACP with Δ9 desaturase. In cat's claw the major FA exporting the plastid is $^{\Delta 9}$16:1, with the minor elongation product being $^{\Delta 11}$18:1 (Chisholm and Hopkins 1964, Cahoon et al. 1997a). Examples of desaturases, other than Δ9 desaturase, include the soluble Δ6 acyl-ACP desaturase that have been detected in the endosperm of *T. alata* (Cahoon et al. 1994) and the Δ4-acyl-ACP desaturase involves the production of $^{\Delta 6}$18:1 of coriander endosperm and transgenic *Nicotiana tabacum* (tobacco) cells (Cahoon et al. 1992, Cahoon and Ohlrogge 1994). Cahoon and Ohlrogge (1994) report that the Δ4 desaturase first used 16:0-ACP as a substrate and the product was elongated to $^{\Delta 6}$18:1-ACP.

In spite of the difference in functional properties these enzymes share, they have ≥ 70% amino acid sequence similarity. The notable difference between the $^{\Delta 9}$18:0-ACP desaturase and the others mentioned include variations in the number of amino acids present in the N-termini (Cahoon et al. 1997a). For example, the $^{\Delta 4}$16:0-ACP desaturase of coriander and the $^{\Delta 9}$16:0-ACP desaturase from milkweed contain 15 and 22 fewer amino acids in the N-terminus region, respectively, relative to the known $^{\Delta 9}$18:1-ACP desaturase sequence from castor bean (Cahoon et al. 1992). Although the number of amino acids in the N-terminus was thought to be associated with substrate specificity, Cahoon et al. (1997a) found that this was not the case for *T. alata*. To test this, the $^{\Delta 9}$18:0-ACP desaturase was compared to a mutant enzyme missing the first 31 amino acids from the N-terminus. The mutant was made according to a prediction from the $^{\Delta 9}$18:0-ACP castor desaturase because 32 amino acids from the N-terminus were thought to influence the substrate specificity. Results of the substrate specificity of the mutant desaturase showed that there was almost no difference in relative activity, and the mutant had only half the specific activity when compared to the WT *T. alata*. Cahoon et al. (1997a) suggested that the N-terminus of the $^{\Delta 9}$18:0-ACP desaturase contributed to the folding or structural stability of the enzyme rather than to binding specificity.

After unsaturation at the plastidial level using a soluble desaturase, further desaturation can occur using glycerolipid desaturases. The unsaturation of glycerolipids is essential for proper function of the biological membranes. At physiological temperatures, polar glycerolipids containing only SFAs cannot form a bilayer needed for membrane function (Los and Murata 1998). The introduction of USFAs enables

membrane fluidity to change from a more solid or gel phase to a liquid crystalline phase at lower temperatures.

There are a number of common glycerolipid desaturases. One includes the Δ12 desaturase that works from the carboxyl end, or ω-6, working from the methyl end, and allows the conversion of $^{\Delta 9}$18:1 to $^{\Delta 9,12}$18:2. The genetic loci responsible for both ER and plastidial Δ12 desaturase include *fad2* and *fad6*, respectively, and have been characterized within *A. thaliana*, spinach, soybean, *Petroselinum crispum* (parsley), *B. rapa* and *Gossypium herbaceum* (cotton) (Pirtle et al. 2001). Other plants only have Δ12 ER glycerolipid level desaturases. For example, *Sesamum indicum* L. (sesame) has two copies of *fad-2* genes (Jin et al. 2001), whereas sunflower has three *fad2* genes (Martinez-Rivas et al. 2001).

Other major and minor glycerolipid desaturases include the Δ15 and Δ6 desaturases, respectively. The major triunsaturated FA in plants is $^{\Delta 9,12,15}$ 18:3, whereas the production of γ-linolenic acid ($^{\Delta 6,9,12}$ 18:3) in plants is uncommon. Known sources of $^{\Delta 6,9,12}$ 18:3 include *Borago officinalis* (borage), *Oenothera biennis* (evening primrose) and the fruits of *Ribes nigrum* (black current) with 26, 9, and 19% of the total FA accounted for by $^{\Delta 6,9,12}$ 18:3, respectively. The production of PUFAs include successive desaturations of $^{\Delta 9}$18:1 to produce $^{\Delta 9,12}$ 18:2 by a Δ12 desaturase and then a second desaturation by a Δ6 or Δ15 to produce $^{\Delta 6,9,12}$ 18:3 or $^{\Delta 9,12,15}$ 18:3, respectively (Galle et al. 1993). The Δ6 or Δ15 desaturases are a source of EFAs in human nutrition. The genes controlling ω-3 desaturases in both *B. napus* and *A. thaliana* have been identified as *fad3* (Arondel et al. 1992), and the *A. thaliana* sequence was cloned using T-DNA (Yadav et al. 1993). Reed et al. (2000) performed the characterization of the $^{\Delta 9,12}$18:2 of

B. napus' extraplastidial desaturase by expressing it within a yeast system. The results included a ω-3 instead of a Δ15 double bond regioselectivity. Also, the desaturase had the ability to act upon carbon lengths of 16 to 22 with a preference for substrates with a ω-6 instead of ω-6 hydroxyl groups, or ω-5 or ω-9 double bonds. Lastly there was insensitivity to double bonds proximal to the carboxyl end.

Although the major desaturases were mentioned above, there are also a number of more rare desaturases that are specific to certain plants. Examples of these include the 1,4 acyl-lipid desaturases in pomegranate seeds (Hornung et al. 2002), and the 1,4 desaturase of *Calendula officinalis* (Reed et al. 2002).

The last level of desaturation in plants, and only very recently discovered, occurs at the level of the acyl-CoA. The first acyl-CoA like desaturase included a *Limnanthes* acyl-CoA Δ5 desaturase gene expressed in soybean (Cahoon et al. 2000). Another example of this includes the cloning and first functional characterization of the Δ9 desaturase from *Picea glauca* (white spruce) acting as a membrane bound acyl-CoA-like desaturase (Marillia et al. 2002).

2.4 Approaches to Alter the Degree of Unsaturation in the Fatty Acyl Groups of Oil Seeds and Plants

2.4.1 Conventional Breeding

Examples of plant breeding to alter the FA composition of oils include the development of both high $^{\Delta 9}$18:1 sunflower varieties for better frying properties and low $^{\Delta 13}$22:1 rapeseed for human consumption (Topfer et al. 1995). In the 1960s, a new type of *B. napus* was bred with a small amount of $^{\Delta 13}$22:1 and glucosinolates. The line was

named canola, and became a prime edible oil. Although the prior example deals with low $^{\Delta 13}$22:1, classical breeding is often used to increase the amount of $^{\Delta 13}$22:1 in oil. There is also requirement for industrial oils containing high amounts of $^{\Delta 13}$22:1, which are used in manufacturing plastic films, high temperature lubricants, surfactants, plasticizers, nylon and surface coatings (Katavic et al. 2001). The highest proportion of $^{\Delta 13}$22:1 in *B. napus* seed oil that can be theoretically achieved via traditional breeding is 66 mole% (Katavic et al. 2001), whereas genetic engineering can result in $^{\Delta 13}$22:1 levels higher than 66 mole%, see section 2.4.5.

2.4.2 Chemical Mutagenesis

Chemical mutagenesis has been used to increase the amount of $^{\Delta 9}$ 18:1 in canola (Auld et al. 1992). Despite the nutritional necessity of PUFA (see section 2.1), oils containing high amounts of PUFA are more prone to oxidative damage than oils with little or no $^{\Delta 6,9,12}$ 18:3. For example, the oxidation rates of $^{\Delta 9,12}$ 18:2 and $^{\Delta 6,9,12}$ 18:3 are 10 and 25 times higher, respectively, than for $^{\Delta 9}$18:1 (Lands 1997). To obtain oils with low PUFA, *B. napus* was treated by chemical mutagenesis via exposure to ethyl methanesulfonate (EMS) and mutants were subsequently selected by the altered ratios of $^{\Delta 9,12}$ 18:2/$^{\Delta 6,9,12}$ 18:3 by rapid screening using the thiobarbituric acid test (Auld et al. 1992). EMS is the most common chemical mutagen for *in vitro* mutagenesis (van Harten 1998). Results of the EMS-induced mutation of *B. napus* resulted in a $^{\Delta 9}$ 18:1 content of over 80% with PUFAs accounting for less than 6%, whereas in the control plants, 60% of the FA was accounted for by $^{\Delta 9}$18:1 and 6.6% from PUFA. The change in FA profile was the result of a mutation in the *fad*2 gene, a gene responsible for the desaturation of 18

carbon chain lengths including 18:1 to 18:2 and 18:3. Mollers and Schierholt (2002) found similar results, except there was also a reduction in the content of both SFA including 16:0 and 18:0. Lastly, Lightner et al. (1994) investigated mutants of the *fad*2 gene in *A. thaliana* finding a 2 – 3 fold increase in the amount of 18:0 present in the seeds and also a significant increase present in the leaves.

2.4.3 Site Directed Mutagenesis (SDM)

SDM involves substituting critical amino acids present in the polypeptide chain that affect enzyme performance. SDM has been used to change the substrate specificity of a desaturase enzyme enabling a different FA to be active in the accommodated site, thereby resulting in a convenient way to produce unusual oils in plants. An example of SDM was used by Cahoon et al. (1997b) to investigate the differences between $^{\Delta 6}$16:0 and $^{\Delta 9}$18:0 desaturases. The group compared the sequences of a $^{\Delta 6}$16:0 and a $^{\Delta 9}$18:0 desaturase and changed the amino acid residues in *T. alata* including alanine 181 to threonine, alanine 200 to phenylalanine, serine 205 to asparagine, leucine 206 to threonine and glycine 207 to alanine. The modified $^{\Delta 6}$16:0-ACP desaturase acted as a $^{\Delta 9}$18:0-ACP desaturase. Although this mutant still acted upon 16:0-ACP after SDM, the ratio of substrate specific activity of 16:0-ACP to 18:0-ACP decreased one to four fold after mutagenesis. In another study, Whittle and Shanklin (2001) engineered a $^{\Delta 9}$16:0-ACP desaturase from a $^{\Delta 9}$18:0-ACP desaturase in castor bean. To change the substrate specificity, two amino acid positions at the base of the substrate-binding cavity were changed including threonine 117 to arginine and glycine 188 to leucine. The mutant $^{\Delta 9}$16:0-ACP desaturase activity yielded a turnover rate that approached that of the

endogenous WT $^{\Delta 9}$18:0-ACP desaturase. This improvement towards a 16C substrate was increased 82 fold from that of the WT.

SDM has also be used to increase oxidative stability, as in the study on *Arachis hypogaea* L. (peanut), where changing aspartate 150 to asparagine caused a reduction in the $^{\Delta 9}$18:0-ACP desaturase activity and created a high 18:0 peanut oil (Bruner et al. 2001).

Lastly, SDM has been used to show small differences between the hydroxylase and desaturase enzymes. Here, the homology between the oleate hydroxylase from *Lesquerella fendleri* has approximately an 81% sequence identity when compared to *A. thaliana* desaturase and about 71% sequence identity to oleate hydroxylase from castor bean (Broun et al. 1998b). The conservation of seven residues in a number of desaturases was assessed when SDM was employed to change the seven residues to their corresponding residues found in *L. fendleri* hydroxylase. The resulting mutant was called m_7LFAH12. Broun et al. (1998b), also replaced the seven residues in the *A. thaliana* fad2 $^{\Delta 9}$18:1 desaturase with the corresponding *L. fendleri* hydroxylases residues and called the mutant, m_7FAD2. The WT and mutants were expressed in yeast cells. The WT yeast cells did not accumulate diunsaturated FAs or hydroxylated FAs, whereas the expression of the FAD2 caused an accumulation of 4% diunsaturated FAs and no hydroxylated FAs, while WT *L. fendleri* accumulated 1.4% diunsaturated FAs and 1.5% ricinoleic acid verifying the dual function of the enzyme. The mutant m_7LFAH12 showed major increases in desaturase activity and a reduction in hydroxylase activity. In the cells expressing the m_7FAH12, the ratio of 18:2 FA to ricinoleic acid was 43 fold that of the cells expressing LFAH12, and there was also a 16 fold increase in the ratio of 16:2

diunsaturated FAs compared to ricinoleic acid in WT *L. fendleri.* The m_7FAD2 mutant accumulated ~0.5% ricinoleic acid with ~50% reduction in diunsaturated FAs. A similar study verifying the close association of hydroxylases and desaturases was conducted by van de Loo et al. (1995), except here the *L. fendleri* oleate 12-hydroxylase from castor bean was transformed into tobacco using a *35S* promoter and resulted in the accumulation of 12-hydroxyoleic acid, suggesting that FA desaturases and hydroxylases share a similar reaction mechanism and evolution. Thus, SDM is a promising method to change the FA profile in plants.

2.4.4 Post Transcriptional Gene Silencing (PTGS)

PTGS enables the expression of genes to be down regulated during oil synthesis in developing seeds, without altering their expression in other parts of the plant (Lui et al. 2001). PTGS expresses a DNA sequence that is complementary to the whole or part of the appropriate target FA biosynthesis gene, and can be inserted either in the forward (sense) or reverse (antisense) orientation. The introduction of the complementary DNA sequence leads to the formation of double stranded RNA (dsRNA), triggering the degradation of this dsRNA and also the complementary mRNA transcripts of the endogenous target gene. The degradation of the mRNA prevents the synthesis of the protein and causes an alteration in the FA biosynthesis enzymes. More recently, the discovery of hairpin RNA (hpRNA) has been used and inserted into constructs to generate a hpRNA containing regions of dsRNA causing a high proportion of gene silencing (Lui et al. 2002).

The first success at manipulating the degree of desaturation in *B. napus* was by Knutzon et al. (1992) via PTGS, where the increase in the amount of 18:0 occurred at the expense of $^{\Delta 9}$18:1. This was accomplished by down regulating the $^{\Delta 9}$ 18:0-ACP desaturation activity via antisense expression of $^{\Delta 9}$ 18:0-ACP desaturation cDNA. The amount of desaturase protein was dramatically reduced resulting in up to 40% of the FA content of the oil being 18:0, making the oil a prime substitute for cocoa butter (Knutzon et al. 1992). Similarly, PTGS has been used to down regulate Δ12 desaturase that converts $^{\Delta 9}$18:1 to $^{\Delta 9,12}$18:2 producing high $^{\Delta 9}$18:1 soybean and *B. napus* oils (Lui et al. 2001). The hpRNA PTGS method has also been employed a great deal in the production of high 18:0 and $^{\Delta 9}$18:1 cottonseed oils, which are used for deep frying (Lui et al. 2002).

2.4.5 Plant Transformation

As pointed out earlier, the FA content of seed oil can change drastically while the composition of the plant membranes are conserved. This suggests that TAG is tolerant to changes in chemical structure and will not perturb the plant's physiology, and allows for a large production of oil containing a manipulated FA content for both food and non-food usage.

Changing the unsaturation in plant oil seeds via genetic engineering has included manipulating FA biosynthetic or TAG bioassembly enzymes. The genes being transferred into the plant includes sources such as cyanobacteria, fungi, mammal, other plants and yeast. The goal includes up-regulating or down-regulating a gene causing a change to the transformed plant's FA composition by either an increase/decrease of an endogenous FA or making an unusual FA in the transformed plant. Note that

manipulating the amount of unsaturation in seed oils is not always performed to reduce the amount of SFA, but can often be used to increase the amount of SFA in the case of producing industrial oils.

Many studies focus on decreasing the amount of SFA, and often this is an attempt to produce a healthier edible oil. A major focus of research to increase USFA content has focused on the increase in $^{\Delta 6,9,12}$18:3 because this FA is a precursor for the synthesis of a class of compounds called the eicosanoids and includes $^{\Delta 5,8,11,14}$20:4. The eicosanoids have many functions related to reproduction and regulation of blood pressure. Although $^{\Delta 6,9,12}$18:3 is currently marketed from the seeds of plants such as evening primrose, borage and black currant, these plants are not oil rich and as a result production of $^{\Delta 6,9,12}$ 18:3 in oleaginous plants has been suggested as a possible alternative (Garcia-Maroto et al. 2002). Recently, a group of Marcaronesian plants from the genus *Eschium* (Boraginaceae) were identified among the richest sources of $^{\Delta 6,9,12}$18:3 (Guil-Guerrero et al. 2000). Of these, *E. gentianoides,* contains 28% $^{\Delta 6,9,12}$18:3 in the total FA composition and a close relative, *E. pitardii*, contains a little less $^{\Delta 6,9,12}$18:3, at 22% of the total oil composition. Garcia-Maroto et al. (2002) cloned and characterized the Δ6 desaturase from *E. gentianoides* and *E. pitardii* by expression in yeast and tobacco. Although yeast does not contain the precursor $^{\Delta 9,12}$18:2 to make $^{\Delta 6,9,12}$18:3, tobacco is known to contain$^{\Delta 9,12}$18:2. The transformation resulted in the expression of the Δ6-*E. pitardii* desaturase gene having the highest expression in the developing fruit of the tobacco and also the highest content of $^{\Delta 6,9,12}$18:3. Both $^{\Delta 6,9,12}$18:3 and $^{\Delta 6,9,12,15}$18:4 (octadecatetraenoic acid) were present in the transgenic tobacco, but were not present in

the control tobacco. In the case of the yeast, $^{\Delta 6,9,12}$18:3 was produced only following supplementation with $^{\Delta 9,12}$ 18:2. Another example of the production of $^{\Delta 6,9,12}$ 18:3 includes the investigation by Hong et al. (2002) where *Brassica juncea*, under the control of a *B. napus* napin promoter (seed specific), was transformed with a Δ6 desaturase from *Pythium irregulare*, an oleaginous fungus. The transformation caused the production of three FA including $^{\Delta 9,12}$ 18:2, $^{\Delta 6,9,12}$ 18:3 and $^{\Delta 6,9,12,15}$ 18:4 with 40% of the seed oil represented by $^{\Delta 6,9,12}$ 18:3. Almost all of the $^{\Delta 6,9,12}$ 18:3 was found in TAG, with only a trace amount present in other lipids. Another group examined tobacco transformed with a Δ6 desaturase from borage (Sayanova et al. 1999). In this experiment $^{\Delta 6,9,12}$ 18:3 was present mostly in the TAG portion of the seeds but was also present throughout the plant. This was not surprising even though the promoter used was *35S*, what was surprising was that the Δ6 unsaturated FAs, including $^{\Delta 6,9,12}$ 18:3 and $^{\Delta 6,9,12,15}$ 18:4, did not change the physiology of either the borage or the tobacco plants used in this study. The $^{\Delta 6,9,12,15}$ 18:4 is a highly USFA and is used for industrial purposes such as oil films, special waxes and plastics (Reddy and Thomas 1996). A similar transformation was performed by Cook et al. (2002) using the Δ6 desaturase from borage, but this time the transformation was into *Lycopersicon esculentum* (tomato) and resulted in a significant increase in $^{\Delta 6,9,12}$ 18:3 and $^{\Delta 6,9,12,15}$ 18:4 with other modifications to the FA profile such as a reduction in $^{\Delta 9,12}$ 18:2 and a concomitant increase in $^{\Delta 9,12,15}$18:3. Yet another group, Reddy and Thomas (1996), transformed tobacco with a Δ6 desaturase gene from the cyanobacteria *Synechocystis* and again produced $^{\Delta 6,9,12}$ 18:3 and $^{\Delta 6,9,12,15}$ 18:4.

Another experiment into the investigation of FAs that are beneficial when consumed by humans includes the increased production of $^{\Delta 4,7,10,13,16,19}$22:6. This long chain FA is essential in cell membrane and tissues in the retina and brain of mammals. It is also essential in the growth and development of infants and the maintenance of function in the adult brain. In a study by Qui et al. (2001), a Δ4 desaturase from *Thraustochytrium sp.* was transformed into *B. juncea* under the control of a constitutive promoter producing $^{\Delta 4,7,10,13,16,19}$22:6 in the vegetative tissue when supplied with $^{\Delta 7,10,13,16,19}$22:5. This suggests that $^{\Delta 4,7,10,13,16,19}$22:6 can be produced via Δ4 desaturation and also may be produced on a large scale in oilseed crops.

Yet another example of increasing USFAs includes the investigation of $^{\Delta 6}$18:1, but this time with a focus on industrial purposes. The melting point of $^{\Delta 6}$18:1 is 33°C, and is being considered for the manufacture of solid fats (Broun and Somerville 1999). Chemical cleavage at the number 6 carbon results in the production of 12:0, a major component of detergents and surfactants and also adipic acid, 6:0, a component of nylon 66 (Cahoon et al. 1992). Coriander is a source of $^{\Delta 6}$18:1, and Cahoon et al. (1992) conducted tobacco transformations with a cDNA encoding the 36 kDa coriander desaturase. The transformed tobacco produced $^{\Delta 6}$18:1 and $^{\Delta 4}$16:1 in callus tissue, whereas neither FA was found in the untransformed callus tissue. The increase of $^{\Delta 6}$18:1 in the transgenic callus of 1 - 4% by weight, suggests that other factors may be needed to increase accumulation of different FAs.

Plant transformation can be used to increase oxidative stability of oils and be manipulated to have high MUFAs. Here, the goal is to decrease the amount of PUFAs by manipulating the Δ-12 desaturase. An investigation by Chapman et al. (2001) included

Agrobacterium mediated plant transformations using a binary vector to suppress the expression of the endogenous *fad2* gene in cottonseed. This was done by subcloning a mutant allele of the *fad2* gene from *B. napus* downstream from a seed specific promoter, phaseolin. The results of this transformation caused an increase in $^{\Delta 9}$18:1 ranging from 21 to 30% by weight of the total FA content, compared to 15% present in the WT. Attempts to increase an oils' oxidative stability have also been made using PTGS and are discussed in section 2.4.4.

Genetic engineering work has been performed on Δ9 desaturases. This has been accomplished by the expression of a yeast cytochrome b_5 dependent Δ9 desaturase in tobacco under the direction of a ubiquitous promoter enabling the conversion of 16:0 - CoA to $^{\Delta 9}$16:1 in the ER (Polashock et al. 1992). The yeast Δ9 desaturase transformation caused an increase in the $^{\Delta 9}$16:1 levels in plant tissues. The increases included an oil content of 2 mole% in seeds of transformed plants compared to none present in the control seeds. Another example of an increase in the content of MUFAs of both 16 and 18 carbon chains includes the results of the study by Grayburn et al. (1992). The investigators transformed tobacco with a rat 18:0-CoA desaturase gene. The expression, under the control of a phaseolin promoter, caused increases in leaf $^{\Delta 9}$16:1 and reduced the amount of 16:0, 18:0 and 18:3 in leaf total lipids. The $^{\Delta 9}$16:1 was mainly found in the PC fraction, and due to the fact that PC is a precursor to TAG this is a good indication that the $^{\Delta 9}$18:0-CoA desaturase gene was successfully working within the tobacco plant. Further investigation showed a large MUFA increase, especially $^{\Delta 9}$16:1, in the leaves, stems, and root tissue, with corresponding decreases in 16:0, 18:0 and 18:3 levels (Moon et al. 2000). Although theses changes occurred, there were very little of

these FAs present in the petal or seed. This trend was the same for the $^{\Delta 11}$18:1 because this FA was also not detected in either the petal or seed portion (Moon et al. 2000). Lastly, Rousselin et al. (2002), produced sunflower with a significant reduction in the amount of 18:0 by transformation with a cDNA encoding a castor bean $^{\Delta 9}$18:0-ACP desaturase.

Another reason to try to increase the amount of USFA in plants includes increasing chill tolerance thereby allowing plants to grow in colder climates. An example of this includes the study by Suga et al. (2002). A low temperature inducible *Cholorella vulgaris* C-27 gene, *CvFad3*, was isolated, cloned and expressed in tobacco. The *CvFad3* gene from *C. vulgaris* was chosen because it encodes ω-3 (Δ15) desaturase that enables cells to harden and survive slow freezing down to –196°C (Suga et al. 2002). With expression of *CvFad3*, an increase in the amount of 18:3 would be expected because this desaturase catalyzes the conversion $^{\Delta 9,12}$18:2 to $^{\Delta 9,12,15}$18:3. In the transformation, one out of the ten plants had increases in trienoic FAs. The leaf lipids showed a decrease in 18:2 and increase in 18:3 phospholipid when comparing the transformed plant to the control plants. Although there was an increase in the amount of 18:3 in the phospholipids components of the choloroplast membrane such as PG, monogalactosyldiacylglycerol and digalatosyldiacylclycerol, no other changes occurred, suggesting the desaturase activity was localized within the ER.

Another approach to decrease the chilling sensitivity of a plant includes altering the substrate selectivity of plastidial AT, particularly GPAT, with sensitive plants having SFA in the *sn*-1 position (Slabas et al. 2001a). *A. thaliana* has a plastidial GPAT with a preference for USFAs, whereas *Cucurbita pepo* (squash) does not contain this substrate

selectivity. Murata et al. (1992) studied chilling sensitivity in tobacco plants expressing either the squash AT or the *A. thaliana* AT, resulting in changes in only the FA composition of PG. In the squash AT transformation experiment there was a reduction in the amount of 18:1, 18:2 and 18:3, whereas an increase was seen in these FAs in the transformed *A. thaliana.* As suspected, transformed tobacco plants containing the squash AT were more sensitive to chilling than those with the *A. thaliana* AT. Taking the experiment one step further, Moon et al. (1995) looked at the effect of the unsaturation in the PG fraction of the transformed squash AT in tobacco and tested the thylakoid fractions for high and low temperatures, and also photoinhibition. The transformation resulted in leaves that were more sensitive to photoinhibition and also had longer recovery of photosynthesis than those of the WT. This suggests that the unsaturation in the FA of PG in the thylakoid membrane stabilizes photosynthesis from low temperature photoinhibition by the recovery of the PS II. In a similar set of experiments, the same effects were found except the transgenic plants were produced by introducing a GPAT gene from *E. coli* into *A. thaliana* (Vijayan and Browse 2002, Wolter et al. 1992).

Brough et al. (1996) genetically engineered LPAAT, the AT that controls the FA substrate in the *sn*-2 position. LPAAT cDNA from *Limnanthes douglasii* was inserted into *B. napus* under a napin promoter in an attempt to increase $^{\Delta 13}$22:1 present in TAG. Total FA composition in transgenic *B. napus* revealed that there was no mole% increase in $^{\Delta 13}$22:1. There was, however, an increase in the $^{\Delta 13}$22:1 in the *sn*-2 position compared to the WT. The WT plants contained no $^{\Delta 13}$22:1 in the *sn*-2 position whereas the transgenic *B. napus* had 9 to 28.3 mole%. These results demonstrate that the LPAAT gene from *L. douglasii* encoded an LPAAT enzyme that could be expressed in *B. napus*.

Similar results were obtained by Lassner et al. (1995) when *B. napus* was transformed with a LPAAT from meadowfoam. Again there was no change in the amount of $^{\Delta 13}$22:1 present in the total lipid, although there was an increase the amount of $^{\Delta 13}$22:1 found at the *sn*-2 position. Zou et al. (1997) also transferred an LPAAT gene into *A. thaliana* and high $^{\Delta 13}$22:1 containing *B. napus* except the gene was from *Saccharomyces cerevisiae* (yeast), *SLC*-1, under the direction of a CaMV35S promoter. The transformation resulted in *B. napus* seed oil with $^{\Delta 13}$22:1 ranging from 49.1% to 56.2 weight%, compared to 45.2 ± 0.5 weight% in the non - transformed plants. Transformed plants also increased production of very long chained fatty acids and ranged from 47.2 to 59.6 mole% of seed oil content compared to 38.1 ± 1.5 mole% in controls. There was also an increase in the amount of $^{\Delta 13}$22:1 present in the *sn*-2 position of the TAG in the transformed *B. napus*.

Lastly, overexpression of a DGAT in *A. thaliana* was performed by Jako et al. (2001) where the cDNA encoding the enzyme was inserted into mutant and WT *A. thaliana.* The mutant *A. thaliana* included a mutation present at the locus on chromosome II designated as *Tag1,* causing a reduction in the amount of DGAT produced in the mutant plants. When the mutant *A. thaliana* was transformed with a single copy of the WT *Tag1* DGAT cDNA, the DGAT activity and TAG content was restored. In the case where the WT *A. thaliana* were transformed, DGAT activity increased by 10 - 70% during seed development and mature seed dry weight increased by 9 - 12% dry weight and an increase in PUFAs.

2.5 Plant Transformation using *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is referred to as the genetic engineer of plants and the Ti plasmid of *Agrobacterium* is used as a vector for the transformation of plant cells. *A. tumefaciens* is the bacterium that is responsible for Crown gall disease in plants, which causes tumors to form in dicotyledonous species. As the tumor ages the bacterium dies and the detection of the bacteria becomes impossible. The Ti plasmid is a double stranded circular DNA, around 200 kb in size, and is responsible for the proper opines, which are a novel amino acid and sugar derivative used for energy in bacterium (Walden 1998). In the Ti plasmid of *A. tumefaciens*, four regions of homology have been identified and two of these, the transferred DNA (T-DNA) and the *vir* regions, have been shown to be directly involved in tumor formation. The T-DNA can be present more than one time in the transformed plant, whereas the other two regions are responsible for the plasmid replication and encode for conjugative transfer.

A. tumefaciens mediated cDNA insertion involves a number of steps with both the plant and the bacterium participating (Walden 1988). Initially, chemotaxis and plant cell conditioning occurs as the plant undergoes a response, as if it were wounded, and phenolic derivatives such as acetosyringone are released. The phenolic compounds condition the plant cell to transformation by possibly inducing cell division. Next, the induction of the *vir* genes occur by the expression controlled by at least two regulatory mechanisms, *virA* and *virG* loci. *Pin* loci are also induced when the plant is wounded. Both *vir* and *pin* expression are mediated by the phenolic compounds produced. The *pin* are not required for virulence, but the *virA* protein is known to be a membrane bound sensory molecule that interacts with *virG* molecule, and together they induce the

transcription of *vir* and *pin* loci. The following step involves the expression of the *vir* loci. Here, the activated *virA* product causes the *virG* protein to induce transcription of many *vir* genes including *B*, *C*, *D*, *E*, and *G*. Although the *vir* loci *A*, *B*, *G*, *D* are critical in tumor formation, the *virC*, *E*, and *F* are not. After the activation of transcription occurs by *vir* loci, the production of T-DNA intermediates occurs. First nicks appear at about twelve hours after exposure to acetosyringone and occur in the border of the T-DNA and also in the T-strand DNA. This strand appears as a single stranded DNA that corresponds to the bottom strand of the T-DNA. There is one copy of a T-strand in every bacterium which is thought to act as the T-DNA transfer intermediate and may arise from the strand displacement following DNA intermediates. The overproduction of the Ti plasmid seems to maximize the level of the T-strand intermediates produced in the infection. In the next step, the transfer of the DNA to the plant cell occurs. Although the exact mechanism by which DNA is transferred to the plant cell is not known, the T-strand DNA is thought to be transferred in an analogous manner to bacterial conjugation. The transfer involves one strand of DNA, protected by the DNA-protein complex, to be transferred from the donor to the recipient bacteria with the 5' end leading. A single bacterium can transfer more than one copy of the T-DNA during the transfer randomly into the plant's genome. Lastly, the stabilization of the T-DNA in the nucleus of the transformed plant cell occurs, followed by the expression of the T-DNA in the plant. Although T-DNA can be integrated stably into the plant genome, it often cannot be expressed, which is thought to be a result of methylated DNA in the transformed tissue.

2.5.1 The Transformation of *A. thaliana* and *B. napus* with a cDNA Encoding Cat's claw $^{\Delta 9}$16:0-ACP Desaturase

Transformation of *A. thaliana* and *B. napus* with cDNA encoding cat's claw $^{\Delta 9}$16:0-ACP desaturase has been performed by Dr. Saleh Shah of the Alberta Research Council in Vegreville, Alberta (Shah and Weselake, unpublished results). For *A. thaliana* transformation cDNA was inserted between the CaMV35S promoter and the polyA signal from the terminator of the rubisco small unit polyadenylation (rbcS-E9) of pea in the pKYLZ71 binary vector (Schardl et al. 1987). The pKYLX71 binary vector also contains the nos-Aph (3') II-nos gene for kanamycin resistance marker. The construct was named pdesC$_1$ and was successfully mobilized into *A. tumefaciens* strain C58 by triparental mating and was used for the transformation of *B. napus*.

There were changes in the transformation vector before *B. napus* was performed. For the transformation of *B. napus*, the CaMV35S promoter was replaced with the seed specific promoter from *L. fendleri,* a bifunctional oleate 12-hydroxylase promoter (LFAH12) (2.2kb), followed by the cDNA from cat's claw $^{\Delta 9}$16:0-ACP desaturase gene downstream of the promoter. Previously, Broun et al. (1998a) showed that the fusion of the LFAH12 promoter to the β-glucuronidase (GUS) coding sequence resulted in a high level of early seed specific expression of GUS activity in transgenic *A. thaliana*. This construct was named pdesC$_2$. The plasmid is shown in Figure 5 and the entire sequence of the pdesC$_2$ vector is shown in Figure 6. The $^{\Delta 9}$16:0 - ACP desaturase sequence from cat's claw is 1560 nucleotide base pairs (bp) in length and encodes both the transit peptide and desaturase sequences. The desaturase and transit peptide sequence is shown in Figure 6. The chloroplast transit peptide present in the cat's claw desaturase sequence

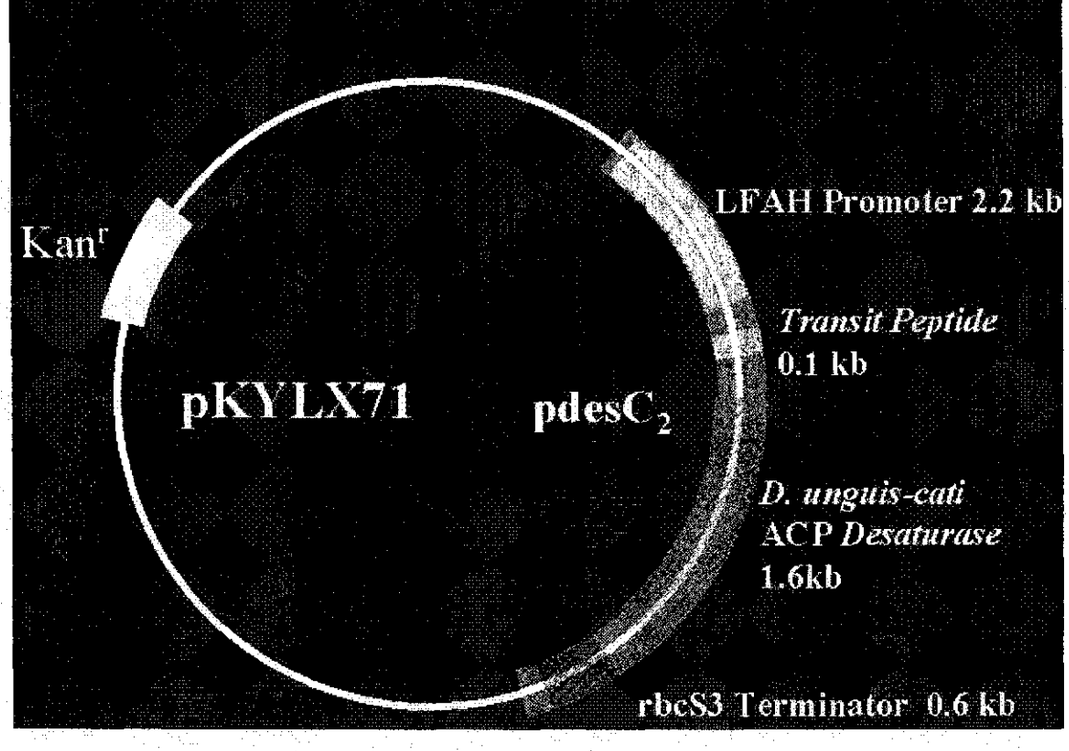

Figure 5. The pdes C_2 expression cassette. The vector was incorporated in a kanamycin resistance pKYLX71 plasmid that contained a 2.2kb LFAH seed specific promoter, a 0.1kb transit peptide, the cDNA encoding the 1.6kb *D. unguis-cati* desaturase and a 0.6kb rbcS3 terminator.

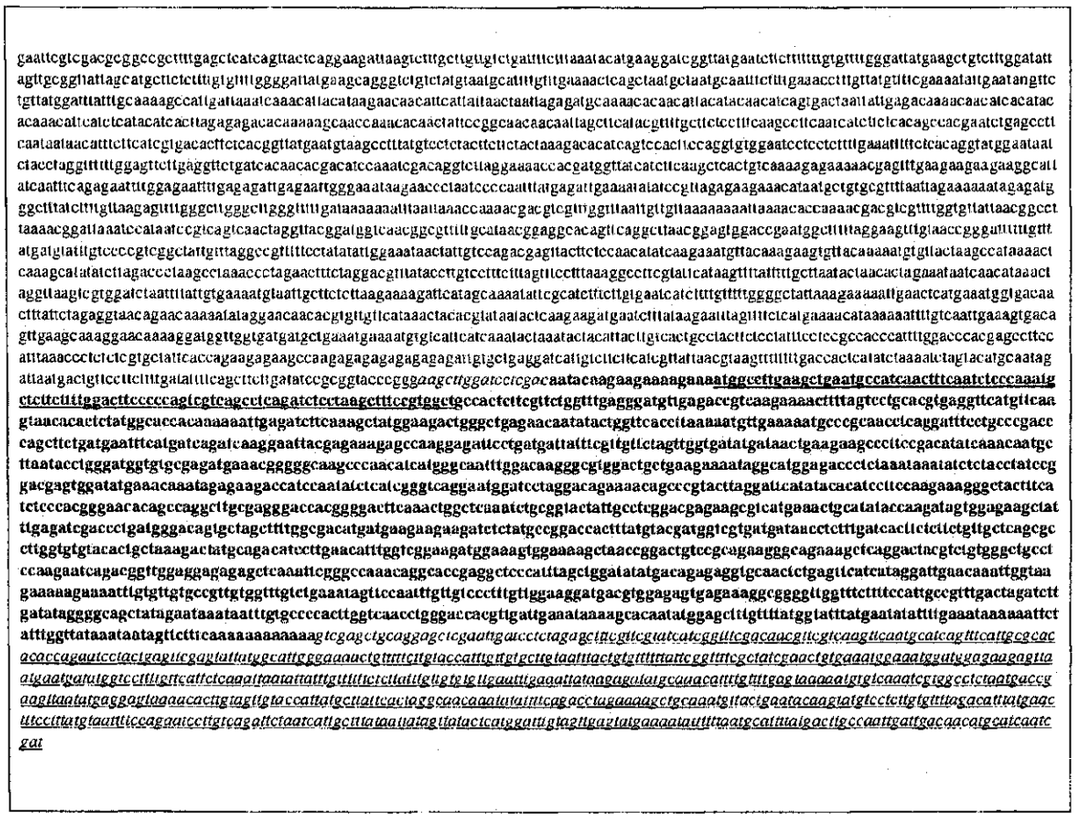

Figure 6. The pdes C_2 vector sequence. The vector is equipped with *(i)* a 2.2 kb seed specific bifunctional oleate 12-hydroxylase promoter from *Lesquerella fendleri;*LFAH *(ii) the multiple cloning site of pKYLX71 (iii)* **the 1.6 kb desaturase gene of *Doxantha*; start and stop codons and the 0.1 kb transit peptide sequence** *(iv) and the 0.6 kb terminator sequence of pea rbcS3 gene*.

was predicted using program the CHLOROP (http://www.cbs.dtu.dk/services /ChloroP) and was shown to be 34 amino acids long. This sequence directs the desaturase into the plastid and is then cleaved off. As cat's claw has a $^{\Delta 9}$16:0-ACP desaturase, it is logical that $^{\Delta 9}$16:1 is the main seed oil present. The FAs present in the seed oil of cat's claw include 16:0 (12%), $^{\Delta 9}$16:1 (64%), 18:0 (<1%), $^{\Delta 9}$18:1 (4%), $^{\Delta 11}$18:1 (15%), and 16:2 (1%) (Chisholm and Hopkins 1964). The cat's claw $^{\Delta 9}$16:0-ACP desaturase sequence has been compared to that of the castor bean $^{\Delta 9}$18:0-ACP desaturase sequence and was found to be 85% identical with a 90% similar amino acid sequence (Cahoon et al. 1998). Also, a single amino acid substitution in the castor bean sequence of leucine 118 to tryptophan caused an 80 fold increase of substrate specificity to 16:0-ACP indicating that the 16:0 and 18:0 desaturase sequences are very similar.

3.0 Materials and Methods

3.1 Chemicals

All solvent used in the Gas liquid Chromatography (GLC) were ordered from VWR Canlab (Mississauga, ON). All the ^{32}P-labelled compounds used were from Amersham Canada Ltd. (Oakville, ON). All other chemicals used were of highest purity available and were from Sigma-Aldrich Canada (Oakville, ON) unless otherwise stated.

3.2 Transformation of Plants

The $^{\Delta 9}$16:0-ACP desaturase cDNA was isolated from cat's claw and cloned by S. Shah into the transformation vector (Cahoon et al. 1998). Transformation of *A. thaliana* ecotype WS and *B. napus* cv.L. Westar were performed via *Agrobacterium* mediated plant transformation, with the pKYLX71 transformation vector (Schardl et al. 1987). The pKYLX71 vector was 12 kb in total and also contained the kanamycin resistance gene.

3.2.1 Transformation of *A. thaliana* Containing Cat's claw $^{\Delta 9}$16:0-ACP Desaturase

The expression cassette was denoted as pdesC$_1$ and included *(i)* a CaMV35S promoter *(ii)* a 0.1 kb transit peptide, *(iii)* the 1.6 kb *D. unguis-cat* L. $^{\Delta 9}$16:0-ACP desaturase, and *(iv)* a 0.6 kb pea rbcS-E9. T$_4$ plants were grown at the Alberta Research Council (ARC), and T$_4$ seeds were sent to the University of Lethbridge, where T$_5$ plants were grown.

3.2.2 Transformation of *B. napus* Containing Cat's claw $^{\Delta 9}$16:0-ACP Desaturase

The expression cassette was denoted as pdesC_2 and included *(i)* a 2.2 kb LFAH12, *(ii)* a 0.1 kb transit peptide, *(iii)* the 1.6 kb *D. unguis-cat* L. $^{\Delta 9}$16:0-ACP desaturase, and *(iv)* a 0.6 kb pea rbcS-E9 (Figure 5). T_0 plants were grown at the ARC, and T_1 seeds were sent to the University of Lethbridge. For generation designation of transformed plants see Figure 7.

3.3 Growth of *A. thaliana*

3.3.1 Identification of T_5 Seeds for Further Propagation

Untransformed *A. thaliana* ecotype WS was planted as a control. The transformed T_4 seeds chosen from the transformed lines including $C_1$16-1, $C_1$26-1, and $C_1$27-1 as these lines showed the highest 16:1/16:0 ratios of 0.24, 0.18 and 0.22, respectively. The control *A. thaliana*, WS, had a 16:1/16:0 ratio of 0.

3.3.2 Specific Conditions for *A. thaliana*

The T_4 and control *A. thaliana* seeds were planted in 100 cm^2 pots containing water saturated Metro Mix soil (Greenleaf Products Inc.) and 5 mL of Nutricote 14-14-14 fertilizer (type 140) (Greenleaf Products Inc). Fifty seeds were brushed onto the surface of the soil of each plant pot in a grid and there were five plant pots per transformed line and control. The pots were then covered with saran wrap and put into the greenhouse for 20 d to allow germination with light supplementation of greenhouse lights for a 16 h day/8 h night cycle. Upon observing the first shoots all pots were transferred to a Conviron growth chamber. The WS *A. thaliana* and the transformed plants were kept in

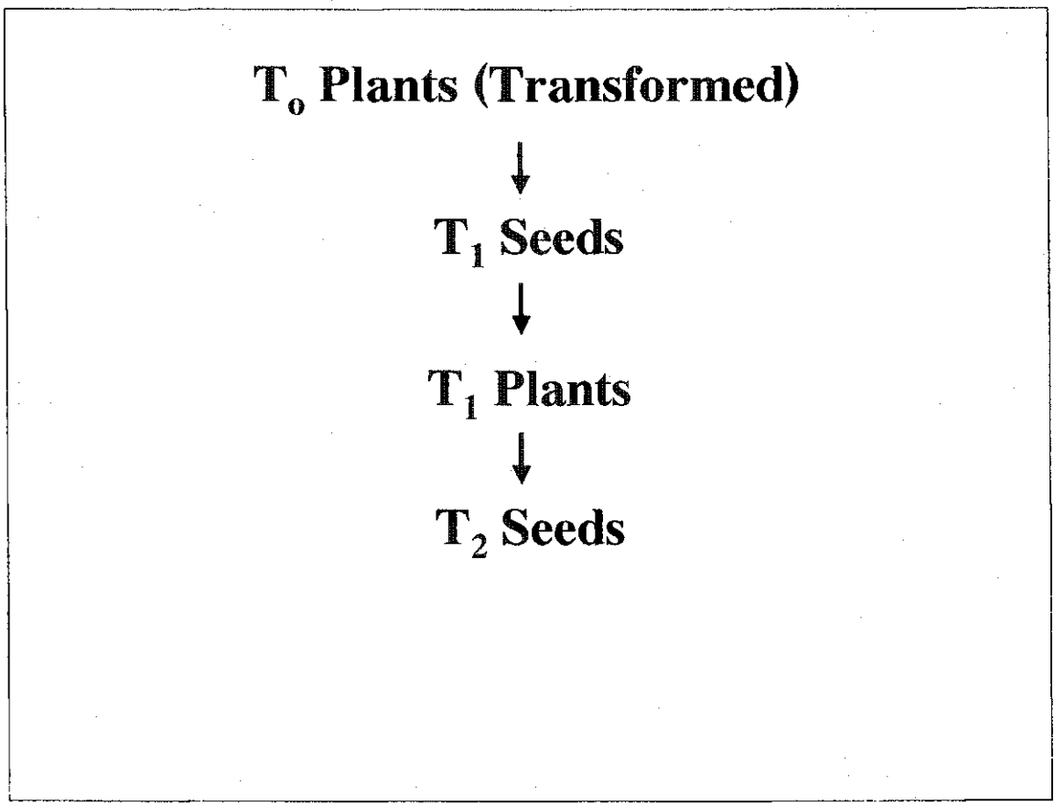

Figure 7. The designation of plant generations for the transformed *B. napus*.

The transformed plants are denoted as T_o and when the T_o plants grow the seeds produced are known as T_1 seeds. The T_1 seeds are then planted and T_1 plants grow to produce T_2 seeds.

separate chambers to prevent cross-pollination of plants. All plants were grown at 23°C and 65 % relative humidity with 16 h day/ 8 h night cycle. The average light intensity during the day cycle was 190 μmol $m^{-2}s^{-1}$. The plants were watered by soaking the bottom of the pots, as needed, to keep soil moist. After 21 d under these conditions, the leaves of all plants were harvested, flash frozen in $N_{2(l)}$ and stored at -20°C until lipid extraction was performed. Following the leaf harvest the plants received no further watering, allowing them to mature. After an additional 20 d of growth under the drought condition the seeds were harvested, frozen in $N_{2(l)}$ and stored at –20°C until lipids were extracted.

3.4 Growth of *B. napus*

3.4.1 Identification of T_1 Seeds for Further Propagation

Untransformed *B. napus* cv. L Westar was planted as a control. The transformed T_1 seeds chosen from the transformed lines included C_2-31A and C_2- 41. These two transformed lines were selected as the analysis of the T_1 seeds showed the highest 16:1/16:0 ratio, 0.10 and 0.11, respectively. Westar control plants had the 16:1/16:0 ratio of 0.04. The T_1 and control *B. napus* seeds were planted in 25 cm diameter pots with water saturated Metro Mix soil (Greenleaf Products Inc.) and 10 mL of Nutricote 14 – 14 - 14 fertilizer (type 140) (Greenleaf Products Inc.). The soil was water saturated again and a single seed was pushed 1 cm into the middle of each pot. All plants were top watered when needed. Once flowering occurred, the plants were covered with bakery bags (Cryovac) to eliminate cross-pollination.

3.4.2 Specific Conditions for *B. napus* -Summer 2001 Plants

Twelve T_1 seeds were planted for each C_2-31A, and C_2-41. Twelve control seeds for Westar were planted and produced seeds to harvest (W1-W12). Of the twelve planted in the C_2-31A line only nine germinated, and only seven of those nine produced siliques for T_2 harvest (C_2-31A-1, 3, 4, 5, 6, 10, 11, and 12). Ten out of the twelve plants in the C_2-41 line germinated and produced siliques for T_2 harvest (C_2-41- 1, 2, 4, 6, 7, 8, 9, 10, 11, 12). All plants were top watered when needed and sprayed with Diazinon (6 mL/L) for Thrips, and once with Ambush (5 mL/L) for Cabbage Pod Weevil. The greenhouse temperature was to be maintained between 23 - 27°C, but often the temperature was as high as 37°C, because of the hot summer and poor ventilation. All records for *B. napus* were kept including anthesis date, amount and time of watering, and spraying. Material collected for experimental work included a) fresh leaf tissue for neomycin phosphotransferase assay (NptII) and Southern blotting, b) micot seeds for RNA extraction c) eight week post anthesis seeds for embryo assay, and d) mature T_2 and control seeds for FA analysis. All mature seeds were collected at around 13 weeks after planting.

3.4.3 Specific Conditions for *B. napus* -Fall 2001 Plants

Fifteen T_1 seeds were planted for each C_2-31A, and C_2-41 also, fifteen Westar were also planted. All seeds germinated and mature seeds from plants were collected (W1 through W15). All fifteen plants in C_2-31A and C_2-41 lines germinated, with only twelve of the C_2-31A line producing siliques and T_2 seeds for harvest (C_2-31A-1, 2, 3, 4, 5, 6, 8, 10, 12, 13, 14, 15,). Of the C_2-41 plants, all produced siliques and T_2 seeds for

harvest (C_2-41-1 through 15). Weekly, the plants were fertilized with Plant Prod Soluble Fertilizer (20/20/20) (Greenleaf Products Inc.) (200 mL of 3 g/L) and moved around the greenhouse to diminish possible plant specific effects related to greenhouse temperature gradients. The greenhouse lights were turned on for a 16 hour light/8 hour dark period to help with the reduction in day light hours. All plants were top watered when needed, and sprayed with Diazinon (6 mL/L) once for Thrips. The greenhouse temperature was to be maintained between 23 - 27°C. The average measured temperature high was 31.34 ± 4.30°C and the average measured temperature low was 15.35 ± 3.28 °C. All records for *B. napus* were recorded including anthesis date, amount and time of watering, spraying, and the temperature high and low for each day. Materials collected included: a) four weeks post anthesis developing seeds for the evaluation of acyl-CoAs (stored at – 80°C), b) mature T_2 and control seeds for Low Resolution Nuclear Magnetic Resonance (LR-NMR) to determine % oil and extract lipids to run on the GLC for FA analysis.

3.5 NptII Detection in Leaf Material from *B. napus* -Summer 2001 Plants

A 1 cm leaf disk was collected in a 1.5 mL microfuge tube. An aliquot of 100 µL of NptII extraction buffer was added (50 mM Na_2HPO_4 pH 7.0, 0.07% β - mercaptoethanol, 10 mM Na_2EDTA pH 8.0, 0.1% Sarcosyl, 0.1% Triton X-100) and the leaf tissue was ground using a teflon bit sterilized in 95% ethanol. The homogenized sample was centrifuged at 13 000 x *g* (4°C) for 20 min and the supernatant was tested for NptII activity against a negative control tube. The negative control tube contained 20 µL supernatant, 10 µL reaction buffer (67 mM Tris-maleate pH 7.1, 42 mM $MgCl_2$, 400 mM NH_4Cl, 1.7 mM DTT) and 10 µL [γ-^{32}P] ATP mix (15 µM cold ATP, 4 µM [γ-^{32}P] ATP

(3 000 Ci/mmol) in reaction buffer) and a test sample containing 20 μL supernatant, 10 μL reaction buffer containing 0.4 mg kanamycin/mL and 10 μL [γ-^{32}P] ATP mix. After incubation at 37°C for 60 minutes, the reaction mixtures were spotted on Whatman P81 paper, and the paper was washed two times with a wash solution (10 mM Na_2HPO_4, 1% SDS) for 30 min at 65°C. The paper was then wrapped in Saran wrap and exposed onto a Multi-purpose phosphoimager film for one hour before an image was taken. The activated film was visualized on a Storage Screen Phosphoimager (Canberra Packard Canada Ltd., Mississauga, Canada). The T_1 leaf tissue included C_2-31A-1, 3, 4, 5, 6, 8, 9, 10, 11, 12, and C_2-41-1, 2, 4, 6, 7, 8, 9, 10, 11, 12. All Westar plants 1 through 15 were also tested.

3.6 Embryo Assay on Developing *B. napus* T_2 Seeds -Summer 2001 Plants

Embryo assay plates (Murashigi and Skoog (MS) Salts with MS vitamins 4.4 g/L (Gibco BRL), 3% sucrose, 2 mg/L 2,4-D pH 5.6, 0.8% agar, 50 mg/L kanamycin, and 0.5 mg/L BA) were prepared and three to four siliques from each plant were gathered and placed in a Flacon tube. All plants that produced siliques were tested (see 3.4.2). The siliques were then sterilized with *(i)* 95% ethanol and drained, *(ii)* a drop of Tween 20 and 70% bleach and then washed for 10 min and drained, *(iii)* 0.025% $HgCl_2$ to cover siliques, washed another 10 min and drained, *(iv)* rinsed four to eight times with d_2H_2O (autoclaved) water and decanted. The siliques were dissected and the embryo was removed using a sterilized scalpel and tweezers to the assay. The embryo was cut into eight pieces and transferred to the media. For each plant being tested, 20 seeds were dissected with 10 embryos on each plate. Once transferred to media, the outside of the

petri dish was taped with micropore tape to eliminate contamination. The plates were then placed in a Revco growth chamber and grown on a 16 h day light/8 h night cycle at 24°C and light intensity between 70 - 80 mmol m^{-2} s^{-1}. T_1 plates where grown from 7 - 14 d until the callus tissue formed and the color of each callus was noted.

3.7 Molecular Genetic Procedures

3.7.1 Small Scale Preparation of Plasmid DNA

Initially, the transfer of a single colony was placed into 5 mL LB medium with tetracycline (15 mg/mL), and incubated at 37°C with vigorous shaking overnight. The following day, 0.5 mL of the culture was pipetted into a microfuge tube and centrifuged at 12 000 x *g* for 5 min at 4°C. The supernatant was removed and the bacterial pellet was resuspend in ice-cold solution #1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) by vigorous vortexing. Next, 200 µL of freshly prepared solution #2 (0.2 M NaOH and 1% SDS) was added to lyse the cells. The tubes were inverted 8 x, and placed on ice for 5 min. When the solution became clear indicating all the bacterial cells are lysed, 150 µL of ice-cold solution #3 (3 M with respect to potassium and 5 M with respect to acetate) was added to precipitate any chromosomal DNA and proteins. The tubes were gently inverted 10 x, and stored on ice for 3 - 5 min. The microfuge tubes were then centrifuged at 12 000 x *g* for 15 min at 4°C, and the supernatant was transferred to a fresh tube. An equal volume of phenol:choloform was added and mixed by vortexing. The tube was centrifuged at 12 000 x *g* at 4°C and the top layer was transferred to a new tube. To precipitate out the double stranded DNA, 2 volumes of 95% ethanol (stored at –20°C) was added, and the mixture was allowed to sit at room

temperature for 2 min. The tubes were then centrifuges at 12 000 x *g* for 15 min at 4°C, the supernatant removed and the tubes were inverted on a paper towel to allow all the fluid to drain. The DNA was air dried to ensure the ethanol was evaporated and the resulting DNA was resuspended in 100 µL of TE with 20 µg/mL of RNase.

3.7.2 Genomic DNA Extraction from Leaf Tissue

Fresh *B. napus* leaf tissue was collected, flash frozen in $N_{2(l)}$, and freeze dried. A mortar and pestle was used to grind 0.3 g of freeze dried leaf tissue with $N_{2(l)}$. A 5.0 mL aliquot of fresh Kirby mix (1% sodium tri-isopropylnaphathalene sulphonate, 6% sodium 4-aminosalicylate, 50 mM Tris-HCL pH 8.0, 6% Tris saturated phenol) was added to the powder. The slurry of leaf powder and Kirby mix was incubated at 65°C for 10 min followed by rocking at 30 rpm for 1 h in a polypropylene Falcon tube. An equal volume of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) was added to the slurry and the mixture was inverted three times and centrifuged at 3 000 x *g* for 10 min to allow phase separation. The upper phase was transferred to a clean tube and at least two other washes were performed each time transferring the upper phase. The washes were repeated until the interface became clear and precipitate formed overnight using 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol. The following day the precipitate was collected by centrifugation at 3 000 x g for 10 min and resuspended in 1.0 mL TE buffer containing 40 µg RNase/mL. Pronase was added to a final concentration of 20 µg/mL and the solution was incubated for 1 h at 37°C. An equal volume of phenol:chloroform:IAA (25:24:1) was mixed into the solution, centrifuged at 3 000 x *g* for 10 min, and the upper phase was precipitated overnight as described above. The

following day, a centrifugation at was performed at 3 000 x *g* for 10 min and the pellet was resuspended in 150 µL d_2H_2O and quantified on a Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech Inc., Baie d'Urfe, Quebec).

3.7.3 Total RNA Extraction from Developing Seeds

Total RNA extractions were prepared on developing T_2 and Westar seeds grown in the summer of 2001 using a TRIzol extraction protocol (Invitrogen Canada). Approximately 1 g of developing seeds were ground with a mortar and pestle using $N_{2(l)}$. Powder was immediately transferred to a 15 mL polypropylene tube containing 10 mL of TRIzol reagent and samples were mixed gently by inversion. Proteins, polysaccharides, lipids and DNA were pelleted by centrifugation at 12 000 x *g* for 10 min at 4°C and the supernatant was transferred to a 15 mL polypropylene tube. Following the addition of 2 mL chloroform and vigorous mixing, the samples were incubated at room temperature for 3 min and centrifuged at 3 000 x *g* for 15 min at 4°C to allow phase separation. The aqueous phase was transferred to new tube containing 2.5 mL of isopropanol and 2.5 mL high salt solution (0.8 M sodium citrate and 1.2 M NaCl pH 7.0). Samples were mixed gently by inversion at room temperature for 15 min, centrifuged at 3 000 x *g* for 10 min at 4°C, and the pellet was washed twice in 75% ethanol (once in 10 mL and the second in 1 mL). During the second wash the pellet was transferred to a 1.5 mL microcentrifuge tube. The supernatant was then discarded and the samples were air dried on a heating block at 60°C. Once the ethanol was removed the pellet was resuspended in 150 µL of DEPC-treated water. Samples were then incubated at 60°C for 10 min, flash frozen in $N_{2(l)}$, and stored at – 80°C.

3.7.4 ^{32}P- Labeled Probes

Probes were made from 1 μg of DNA isolated from gel electrophoresis and the gel was extracted with Qiagen Gel Extracton Kit. The DNA was diluted to a volume of 31 μL and heat denatured for 10 min in boiling water followed by cold shocked on ice for 2 min. To the DNA 10 μL of OLB buffer was added (random hexamers and dNTPs), 2 μL BSA (10 mg/mL), 2 μL Klenow enzyme (2 U/μL) and 5 μL[α-^{32}P]-dCTP (3 000 Ci/mmol). The reaction was allowed to incubate at room temperature for 4 hours at which time 50 μL of stop dye (1% dextran blue, 0.33% bromophenol blue, 13.3% glycerol, 40 mM EDTA, pH 7.6). The reaction mixture was added to a Biogel-60 column (bed volume = 2 cm^3) suspended in 20 x TES (2 mM EDTA, 4% SDS containing 200 mM Tris-HCL buffer pH 7.6). The lower phase was collected in a microfuge tube and heat denatured for 10 min followed by cold shocked as described above.

3.7.5 Southern Blotting

Samples tested were loaded on an 0.8% agarose gel and the DNA was transferred to Hybond – N$^+$ positively charged nylon membrane (Version 2.0) (Amersham Canada Ltd, Oakville, ON). Blotting was performed according to the protocol provided by the supplier. For imaging, the hybridized membrane was wrapped in saran wrap, exposed to a multi purpose film, and visualized on a Storage Phosphor Screen Phosphoimager (Canberra Packard Canada Ltd., Mississauga, Canada).

3.7.5.1 Genomic DNA

A mass of 20 μg of genomic DNA from *B. napus* was restricted overnight with PvuII and EcoRI (New England Biolabs, R0151S and R0101S, respectively) for each plant being tested. The probes used included the $^{\Delta 9}$16:0-ACP desaturase sequence, 1.6 kb, for the analysis of the genomic DNA. See Figure 6 for the entire pdesC_2 sequence.

3.7.5.2 Reverse Transcription –Polymerase Chain Reaction (RT-PCR) Product

A volume of 45 μL of the amplified cDNA was used. The membrane was hybridized twice, once with the amplification product using the 16Des1 and 16Des2 primers and the second with the amplification product of the Tu1 and Tu2 primers.

3.7.6 RT-PCR

A mass of 60 ng of total RNA were reverse transcribed using random primers p$(dN)_6$ and the Roche 1st Strand cDNA Synthesis kit for RT-PCR (AMV Cat. No. 1 483 188) according to the protocol provided. A PCR reaction mixture was setup containing 5 μL 10 x PCR reaction buffer, 1.5 μL 50 mM $MgCl_2$, 1.0 μL 10 mM dNTPs, 1.25 μL 10 μM 16Des1, 1.25 μM 16Des2, 1.25 μL 4 μM Tu1, 1.25 μL 4 μM Tu2, 0.25 μL *Taq* polymerase, 35.25 μL OPTIMA H_2O, and 2.0 μL of RT product. For sequences of the primers 16Des1 and 16Des2 used see Figure 8. The Tu1 and Tu2 primers were designed from the *A. thaliana* sequence for the nuclear encoded mitochondrial elongation factor-Tu (GenBank accession #T04276). The Tu elongation gene was used as a control in the PCR. The forward primer, Tu1, sequence was (5'-GTAGGACTTCTTCTGCGTGG-3') and the reverse primer, Tu2, sequence was (5'-AACTGTTCTACCTCCTTCCC-3'). The

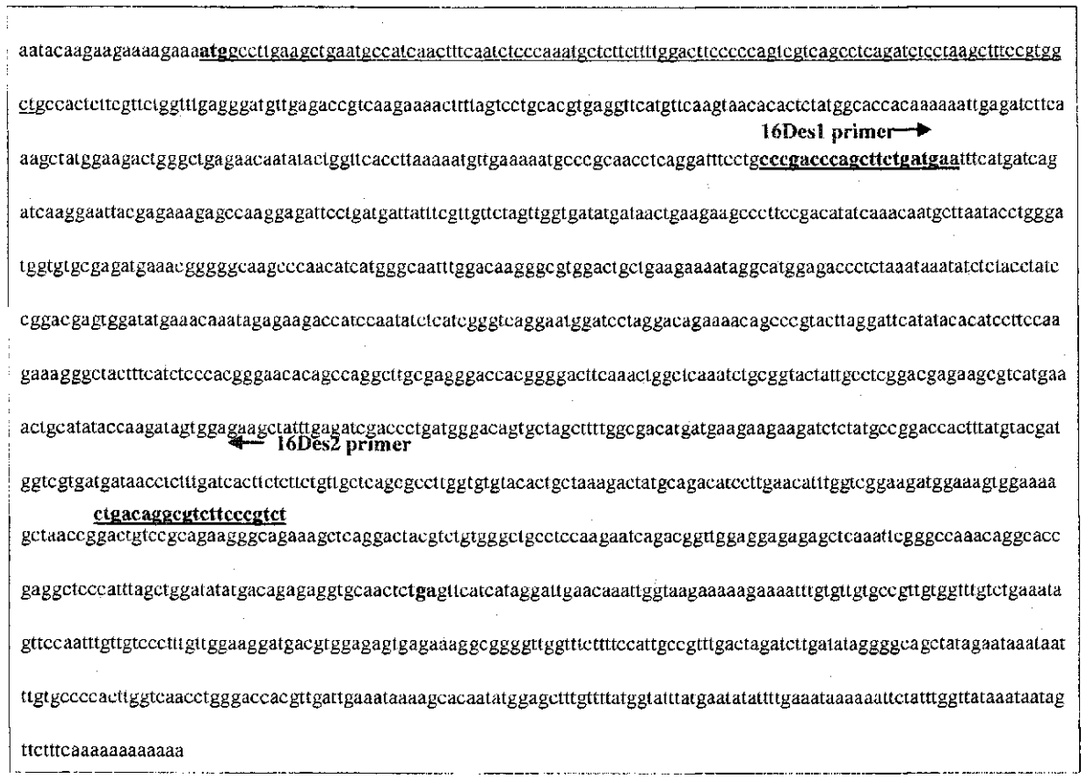

Figure 8. Amplification of $^{\Delta 9}$16:0 desaturase cDNA of cat's claw with 16Des1 and 16Des2 primers. The 1.6 kb desaturase cDNA of cat's claw; **start and stop codons**, the 0.1 kb transit peptide sequence, and **16Des1 and 16Des2**. Both primers are 21 nucleotides in length.

PCR reaction was run for 38 cycles because this amplification was in the linear range; y = 1.65x – 47.75, r^2 = 0.979. The PCR amplification conditions were 94°C for 3 min (hot start), and 38 subsequent PCR cycles at 94°C for 45 sec, 57.6°C for 30 sec (specific for 16Des1 and 16Des2), and 72°C for 90 sec. Following the 38 cycles a final extension of 72°C for 10 min was performed. The amplified DNA was then electrophoresed on a 0.8% (w/v) agarose gel

3.7.6.1 Primer Design and Synthesis of 16Des1 and 16Des2

Primers were designed for the PCR amplification of the $^{\Delta 9}$16:0-ACP desaturase from cat's claw sequence. The forward primer, 16Des1, (5'-CCCGACCCAGCTTCTGAA-3') was designated to anneal to the complementary sequence of cDNA nucleotides 323-343. The reverse primer, 16Des2 (5'-TCTGCCTTCTGCGGACAGTC-3'), will anneal to nucleotides 1062-1083 of the cDNA seqence (figure8). The optimal annealing temperature for the primer combination was 57.6°C, and the PCR product should generate a 760 bp product. The primers were synthesized with a Beckman Oligo 1000 DNA Synthesizer (Beckman Instruments, Fullerton, CA) at the Lethbridge Research Station, Lethbridge.

3.8 Isolation and Analysis of Lipids

3.8.1 Oil Analysis Using LR-NMR of *B. napus* -Fall 2001 Plants

Oil seeds analysis was performed on a mq 20 LR-NMR Analyzer Bruker minispec (Milton, Ontario). The LR-NMR was calibrated with *B. napus* samples from the Grain Research Laboratories of the Canadian Grain Commission. Each sample of

about 3 g was weighed out on an analytical balance to four decimals. The samples run included C_2-31A-1 (n = 12), C_2-41-1 (n = 14), and Westar (n = 14). The LR - NMR tube was placed in the instrument and the % oil was obtained and was corrected for the exact mass. The analysis was performed for each sample listed above in the same LR-NMR 18.2 mm diameter tube.

3.8.2 Lipid Extraction

For all lipid extractions and analysis only glass pipettes and vials with teflon lids were used. As well, all hexane used was HPLC grade. Prior to extraction all glassware was washed with hexane and dried with $N_{2(g)}$.

3.8.2.1 Lipid Extraction using the Hexane Isopropanol (HIP) Procedure

Approximately 30 mature seeds were weighed (~ 0.1 g) when *B. napus* was analyzed, whereas ~0.2g of frozen leaf tissue or seeds were used in the analysis of *A. thaliana.* Tissue being analyzed were placed in a glass homogenizer and one milliliter of isopropanol was added and the tissue was boiled for 10 min at 80°C and then placed on ice for 2 min. Next, 1 mL of each hexane and 3:2 HIP was added and the tissue was crushed. The solution was then transferred to a screw cap vial, the homogenizer was rinsed with 1 mL 3:2 HIP, and the rinse was added to the same collection vial. To the collection vial, 3:2 HIP was then added until the final volume was 6 mL. Next, 2 mL of 3.3% Na_2SO_4 was added, the solution was mixed, and gently centrifuged to separate the phases and the upper organic phase was transferred to a new vial. Another extraction was performed on the original tissue sample using 7:2 HIP and the upper phase was combined

with the previous extract. The contents were dried under $N_{2(g)}$ at 40°C with periodic rinses of 7:2 HIP along the walls of the vial to concentrate all the oil at the bottom of the tube. The oil was removed with 3 washes of 2:1 chloroform:methanol into a preweighed tube and contents were completely dried again under $N_{2(g)}$. The vial was weighed for gravemetric oil content and diluted to 5 mg/mL in hexane. Only *B. napus* grown in summer 2001 and the *A. thaliana* seed and leaf tissues were analyzed by the HIP method.

3.8.2.2 Lipid Extraction using Petroleum Ether (PE) Procedure

Approximately 30 mature seeds were weighed (~ 0.1 g), placed in an electric homogenizer and 1 mL of petroleum ether (b.p 35-60°C) was added and the sample was ground. The solvent was transferred to a clean preweighed vial, and the meal was washed with 3 x 1 mL of petroleum ether combining the washes. The sample was dried down and resuspended to 5 mg/mL in hexane. *B. napus* seeds harvested in both the summer and fall of 2001 were analyzed by the PE extraction procedure.

3.8.2.3 TAG Isolation from Total Lipids in *A. thaliana*

The TAG fraction was isolated from the seed total lipids extracts of *A. thaliana* using 10 x 20 cm^2 thin layer chromatography (TLC) plates, pre-coated HP-TLC Fertigplatten Kieselgel 60 (VWR Canlab, Mississagua, ON. Canada). Fifty μL of 10 mg/mL of the seed total lipid fraction was spotted across the origin of the TLC plate against 25 μL of a 10 mg/mL 19:1 trinonadecenoin TAG standard. The loaded plate was allowed to air dry before it was run in a TLC jar containing 40 mL of solvent consisting of hexane : diethyl ether : glacial acetic acid (80:20:1 v/v/v). The plates were removed

when the solvent front was approximately 2 cm from the top of the plate, and air-dried. The 19:1 standard was visualized using $I_{2(g)}$ and the corresponding area of the *A. thaliana* sample was scraped and using a glass microscope slide. The scraped silica containing the TAG fraction of the total lipid extract was stored at –20°C until the sample was methylated and run on the GLC.

3.8.3 FAs and GLC Analysis

3.8.3.1 Methylation of Fatty Acids with Methanolic HCl (MHCl)

Three milliliters of MHCl were added to a $N_{2(g)}$ dried sample. For the total lipid analysis of *B. napus* grown in summer 2001 and *A. thaliana* 50 µL (5 mg/mL) of sample was used, whereas the entire scraped TAG fraction, containing TAG and silica, were directly methylated. The solution was heated at 50°C for 16 h under $N_{2(g)}$ (Christie 1992). Tubes were removed and cooled. To each tube 0.25 mL of water was added and the fatty acid methyl esters (FAMEs) were extracted twice with 4 mL of hexane. The extracted FAMEs were transferred to new tubes and dried under $N_{2(g)}$ at 40°C. Then 0.5 mL of GC grade hexane was added to each tube and solution was transferred a GC vial, flushed with $N_{2(g)}$, and sealed with a lid (Christie 1992).

3.8.3.2 Methylation of FA with Sodium Methoxide (NaMeth)

Fresh 2% methylating solution (1 g sodium methoxide and 50 mL methanol) was made fresh and 1.2 mL was added to 50 µL (5 mg/mL) of sample dried under $N_{2(g)}$. The reaction sat at room temperature for 30 min. After 30 min, 1.0 mL of d_2H_2O and 2 x 2mL of hexane was added, mixed, and phases allowed to separate. The upper phase from each

hexane addition was removed, dried under $N_{2(g)}$, and resuspended in 0.5 mL GC grade hexane. The sample was transferred to a GLC vial, flush with $N_{2(g)}$, and seal with a lid.

3.8.3.3 GLC Conditions

All samples were analyzed on a flame ionization gas chromatograph (model 5890, Helwett Packard, Mississauga, ON, Canada) with a J&W Scientific 30m DB-23 column (Chromatographic Specialties, Brockville, ON, Canada). The samples were analyzed for the following FAMEs: methyl myristiate (14:0), methyl palmitate acid (16:0), methyl palmitoleate ($^{\Delta 9}$16:1), methyl sterate (18:0), methyl oleate ($^{\Delta 9}$18:1), methyl vaccenate ($^{\Delta 11}$18:1), methyl linoleate ($^{\Delta 9,12}$18:2), methyl linolenate ($^{\Delta 9,12,15}$18:3), methyl arachidate (20:0), methyl behenate (22:0), methyl eurcate ($^{\Delta 13}$22:1), and methyl lignocerate acid (24:0). The standards were purchased as FAMEs from NuCheck Prep.

3.8.3.4 GLC Conditions for Mature Seed FA Analysis

All samples were run on RAMP3A method. This method contained the following parameters: the split injection volume was 3 μL, set temperature was 180°C (5 min) and the temperature was ramped 2°C/min to a final temperature of 230°C.

3.8.4 Isolation and Analysis of Acyl-CoAs

3.8.4.1 Acyl-CoA Extraction and Methylation Procedure of Four Week Post Anthesis Developing Seeds from *B. napus* -Fall 2001 Plants

For each sample a mass of 10 x 100 mg of bulk developing seeds were placed in a polypropylene microfuge tubes and 200 μL of fresh extraction buffer was added (2 mL 2-propanol, 2 mL 50 mM KH_2PO_4, pH 7.2, 50 μL glacial acetic acid, and 80 μL 50 mg/mL

essentially fatty acid free BSA). The sample was ground with a teflon bit sterilized in 95% ethanol. To remove the lipids and pigments 200 μL of petroleum ether (b.p. 40 - 60°C) saturated with 1:1 (v/v) 2-propanol/water was added, and centrifuged at 100 g for 1 min. This step was performed 3 x and each time the upper phase was removed and discarded. To the lower phase 5 μL of saturated $(NH_4)_2SO_4$ and 600 μL of chloroform/methanol (1:2) was added. The tubes were capped, vortexed to mix, and left on the bench at room temperature for 20 min. After 20 min, the tubes were centrifuged at 21 000 x *g* for 2 min. The supernatant of two tubes was applied to a dry alumina column, and the column was washed with chloroform:ethanol (1:2) (v/v). To elute the acyl-CoAs, 0.1 M potassium phosphate, pH 7.2/methanol (1:1) (v/v) was added. To the elutant 50 μL of acetic acid (pH 5) was added and the methanol was evaporated with $N_{2(g)}$. From the aqueous phase the acyl-CoAs were extracted with n-butanol, and five tubes from the extraction were combined together. The n-butanol was evaporated using $N_{2(g)}$. The samples were methylated with 2 mL 5% (w/v) H_2SO_4 in methanol at 75°C for 2 h under $N_{2(g)}$. At the end of 2 h the samples were cooled to room temperature and the FAMEs were extracted by adding 4 mL of d_2H_2O and 3 x 2 mL hexane. The organic phase was removed and combined in a new tube. The hexane layer was washed with 1 mL Na_2CO_3 and the organic phase was extracted and dried under stream of $N_{2(g)}$. The extracted FAMEs from the acyl-CoAs were then resuspended in 50 μL of GLC grade hexane and place in 100 μL volume GLC tube. This procedure was a combination of protocols from Larson & Graham (2001) and den Breejen et al. (1989).

3.8.4.2 GLC Conditions for Acyl-CoA Analysis

The GLC program used to analyze the FAMEs from the extraction of the acyl-CoAs was SPLTLSS8. The program had the following parameters: the splitless injection was 5 μL, the set temperature was 180°C (5 min) and the temperature was ramped 2°C/min to a final temperature of 230°C.

3.8.5 Statistics

Analysis of variance (ANOVA) tests were performed for all statistical analysis of the FAMEs from the extracted lipids of the mature seeds, leaves, and also acyl-CoAs. ANOVA was chosen due to the completely randomized design of all experiments. Significant differences included P - values less than or equal to 0.07 and not the common P - value of 0.05. The slightly higher P - value was chosen because by chance we would expect a P - value to be 0.05, meaning 5% of the time there will be no difference in the mean between the two samples being compared. All statistical analysis was performed using JMP IN statistical software (version 3.2.1, Duxbury Press, Toronto, ON).

4.0 Results and Discussion

4.1. Ubiquitous Expression of a cDNA Encoding Cat's Claw 16:0-ACP Desaturase in *A. thaliana*

The analysis of transgenic *A. thaliana* only included determining the FA profiles from the total seed acyl lipid, seed TAG, and the leaf total acyl lipid. The analysis did not include any molecular work.

4.1.1. FA Composition of Total Acyl Lipid and TAG from T_4 Seeds

Analysis of both the seed total acyl lipid and seed TAG from transgenic *A. thaliana* were investigated to determine if there were any changes to the FA seed profiles as a result of the transformation. The FA composition of the seed total acyl lipids is depicted in Table 1 (in Appendix) and Figure 9. The FA composition of TAG is shown in Table 2 (in Appendix) and Figure 10, whereas the FA composition of TAG compared to FA of the seed total acyl lipids from the transgenic *A. thaliana* to the untransformed WS are in Table 3 (in Appendix) and Figure 11. The mole % of SFAs from the seed total acyl lipids increased in all transgenic lines compared to the untransformed WS, and significantly in the 16-1 and 27-1 lines (Figure 9). The increase in the SFA content was due to significant increases in the SFAs that were equal to and longer than 18:0 (Table 1 in Appendix). In TAG, there were no significant changes in the overall SFAs compared to the WS control due to the high S.E., but there were significant increases in 20:0 in all transformed lines (Table 2 in Appendix). The significant increase in the 20:0 from the total acyl lipids extracted from the *A. thaliana* seeds are probably attributed to the increase in the 20:0 of the TAG fraction.

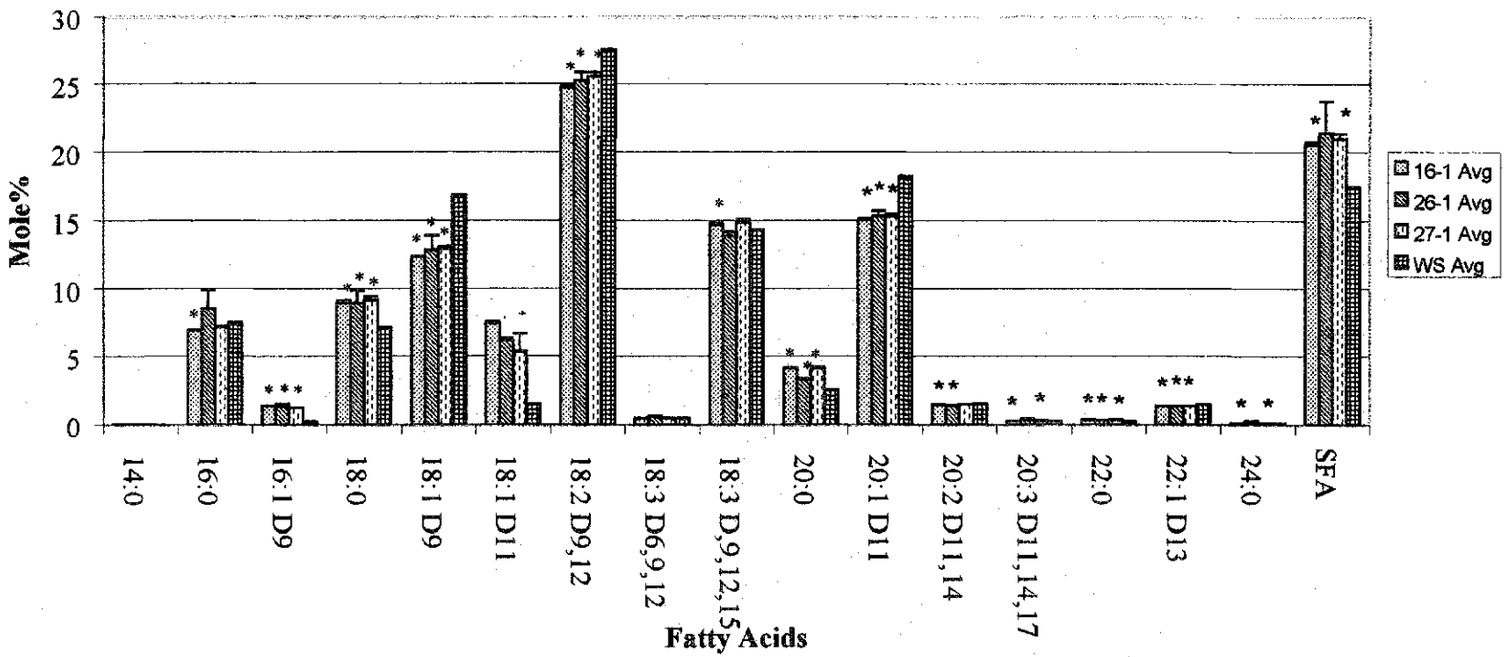

Figure 9. The FA composition (mole%) + S.E. of seed total acyl lipids from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The total acyl lipids were extracted from mature *A. thaliana* seeds using the HIP extraction, methylated by methanolic-HCl and the FAMES were run on a GLC. Transformed lines include 16-1, 26-1, 27-1, and were compared to the untransformed control WS using ANOVA testing. All significant differences are indicated with an * and n=5 for all samples.

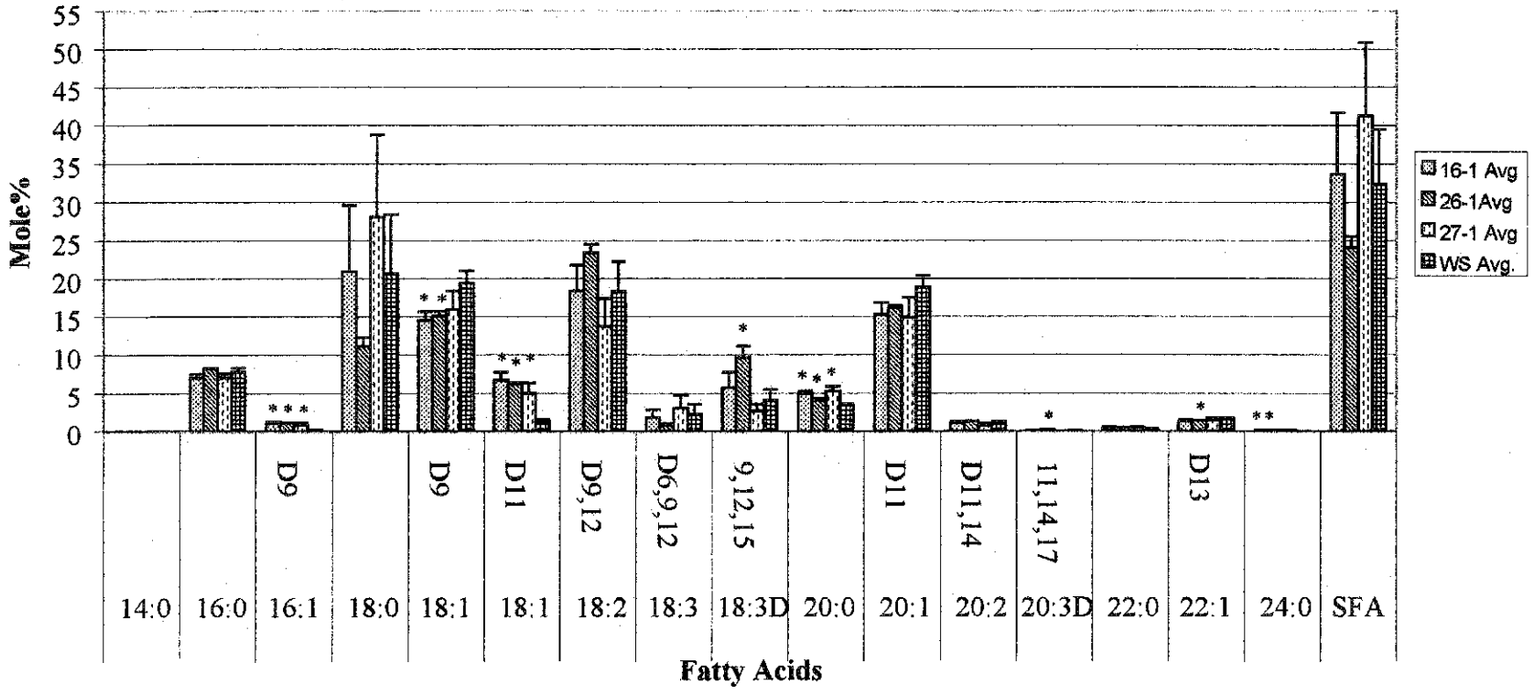

Figure 10. The FA composition (mole%) + S.E. of seed TAG from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The TAG portion was extracted from the seed total lipids, methlyated using methanolic-HCL and FAMES were run on the GLC. The transformed lines, 16-1, 26-1, and 27-1, were compared to the untransformed control WS using ANOVA testing. All significant changes are shown with an * and for all samples n=5.

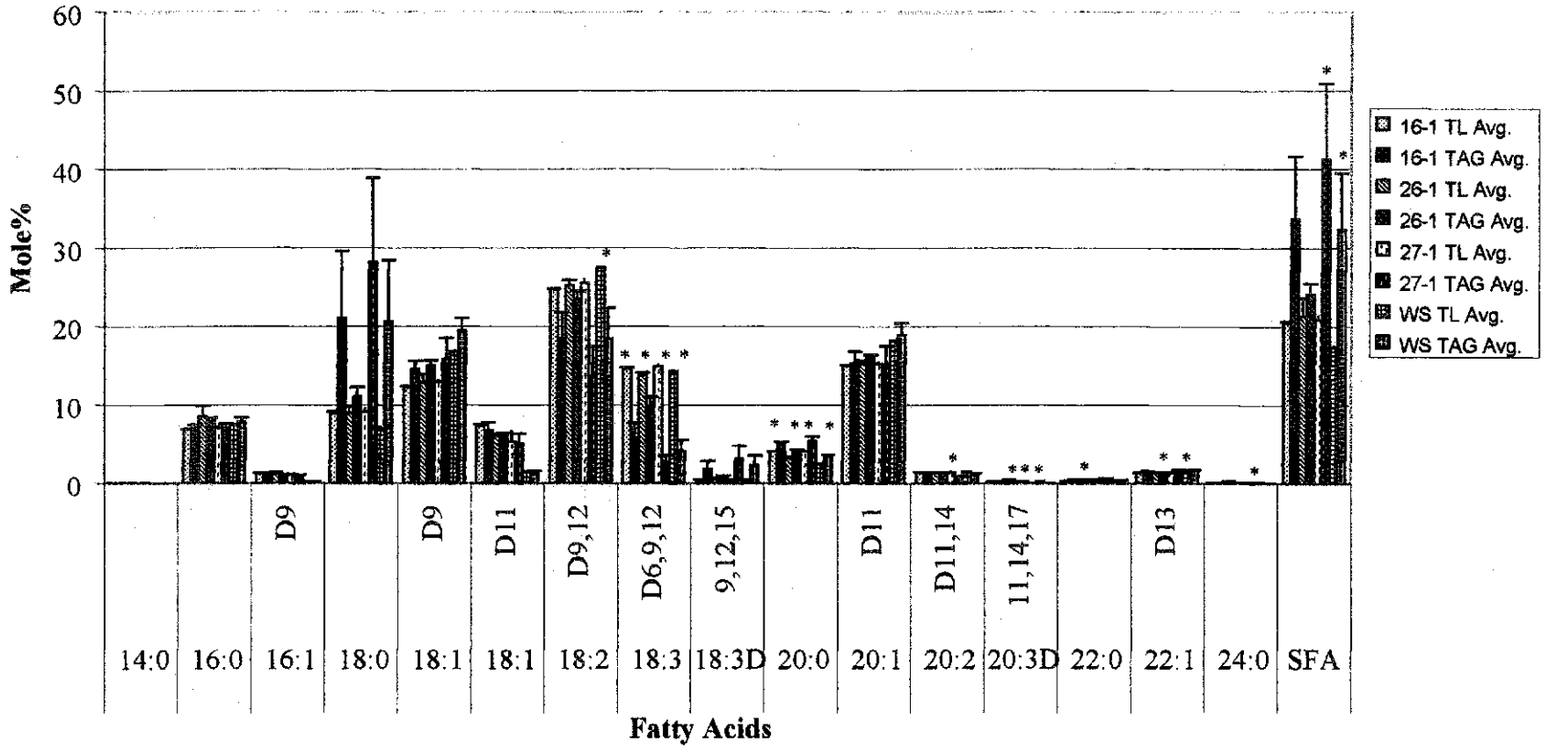

Figure 11. Comparison of the FA composition (mole%) + S.E. of the seed total acyl lipids and TAG from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The lines compared included 16-1 seed total acyl lipids and 16-1 TAG, 26-1 seeds total acyl lipid and 26-1 TAG, 27-1 seed total acyl lipid and 27-1 TAG, and WS seed total acyl lipid and WS TAG fraction using ANOVA testing and all significant differences are indicated with an * and for all samples n=5.

While there were no increases in the amount of USFAs in either the total acyl lipids or the TAG fractions from the seeds of *A. thaliana*, there were significant increases in both the $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1 when compared to untransformed WS. This suggests that the cat's claw 16:0-ACP desaturase was inserted in the *A. thaliana* genome and was being expressed. Also, the significant increases in both $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1, appear to be a result of the incorporation of these FAs into TAG rather than the membrane (Table 3 in Appendix and Figure 11).

When investigating the 16:1/16:0 ratios of the transformed plant lines compared to untransformed, WS, all 16:1/16:0 ratios were significantly increased in the total acyl lipid of the seed, and most of this increase was a result of the contribution from the TAG fraction (Table 4 in Appendix and Figure 12).

To determine if there were differences between the total acyl lipid and TAG fractions from the seeds of the transformed and untransformed *A. thaliana*, the two fractions were compared (Table 3 in Appendix and Figure 11). There were much higher SFAs present in the TAG fractions than in the total acyl lipid, with increases in both 18:0 and 20:0, and decreases in $^{\Delta 6,9,12}$18:3 in TAG compared to total acyl lipid. These differences were not a result of the insertion of the cat's claw desaturase cDNA, because both transgenic and non-transgenic plant lines showed the same differences.

In summary, the cat's claw 16:0-ACP desaturase cDNA appears to have been inserted into *A. thaliana* genome. The significant increase in $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1 in all transgenic lines compared to the untransformed WS in both seed total lipid and TAG fraction further suggest that the cDNA was being expressed. The increase in $^{\Delta 9}$16:1 and

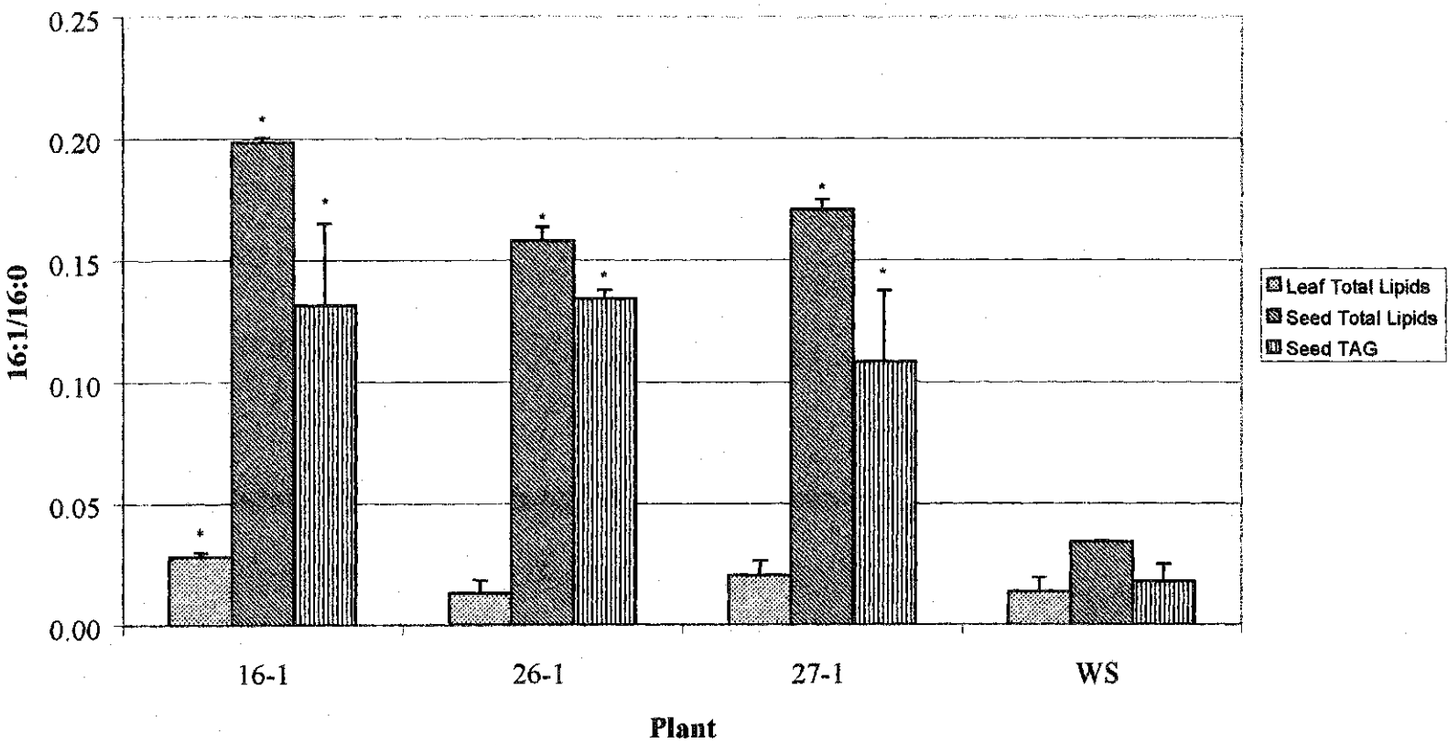

Figure 12. 16:1/16:0 + S.E. of *A. thaliana* transformed with a cDNA encoding a cat's claw 16:0-ACP desaturase. The ratios were determined for leaf total lipid, seed total lipid, and seed TAG fractions. Analysis using ANOVA was performed between the transformed plant lines and the control WS and all significant changes are marked with an *. For all samples n=5.

$^{\Delta 11}$18:1 was mainly associated with TAG. Also, the total acyl lipids from the seeds and the seed TAG from all transgenic lines showed increases in the SFA content

4.1.2. FA Composition of Total Acyl Lipid from Leaves

The purpose of examining the FA profile in the leaves of transgenic *A. thaliana* containing the cDNA for cat's claw desaturase with *35S* promoter was an attempt to determine if the FA profile was increased in USFAs, which could potentially increase the chilling tolerance of genetically engineered plants. At lower temperatures, the fluid state of membrane lipid components is thought to be one of the prerequisites for survival (Wolter et al. 1992), therefore, increasing the USFAs at a lower temperatures should help the maintenance of membrane fluidity. In the current research, the change may be expected to come from a decrease in 16:0 and concomitant increase in $^{\Delta 9}$16:1. It is known that the chilling sensitivity of plants is closely related to the USFAs present in the PG of chloroplast membranes (Murata et al. 1992). Our study focused on total lipids from the leaf. Lastly, although, it is well established that the composition of the FAs in plant membrane phospholipids are temperature-dependent (Caiveau et al. 2001), all plants were grown at 23°C. This allows only the investigation of the differences in the FA composition, due to the effect of transforming with the cDNA encoding the cat's claw desaturase without imposing a low temperature treatment.

The FA profile of total acyl lipids from the transgenic *A. thaliana* leaves did not decrease in SFA content. The levels of SFA were slightly, but not significantly, increased compared to untransformed WS (Table 5 in Appendix and Figure 13). The GLC standard for 16:3 was not available, but was included in the FA profile because 16:3

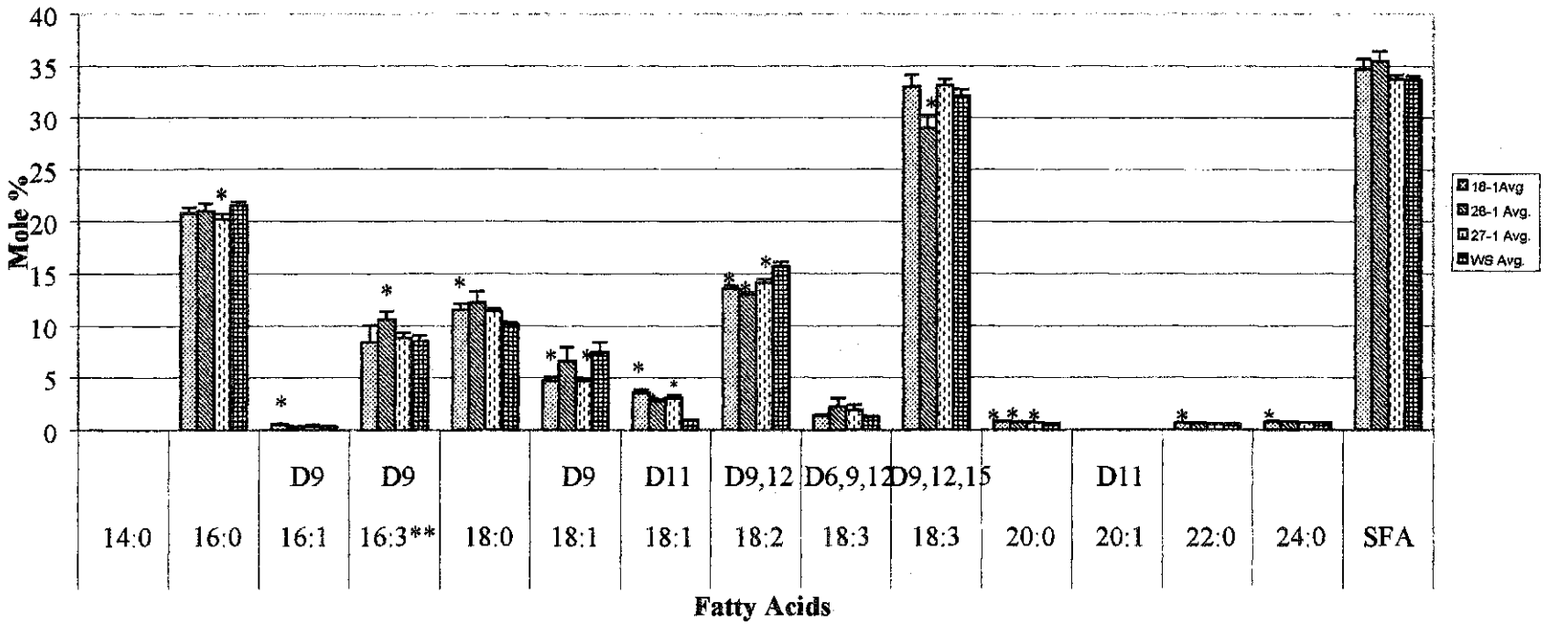

Figure 13. The FA composition (mole%) + the S.E of leaf total acyl lipids from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The total leaf acyl lipids were extracted using HIP extraction, methylated with methanolic-HCl, and FAMES were run on a GLC. Note ** did not have a standard but was assumed to be 16:3, but due to the major content of 16:3 known to be present in *A. thaliana* leaves the FA was included. All significant changes are noted with an * and for all samples n=5. The samples include 16-1, 26-1, 27-1, and WS.

is known to be a major FA present in the leaves of *A. thaliana* (Browse et al. 1989, Kunst et al. 1988, Lemieux et al. 1990). The lack of change in the leaves is not surprising, because both structure and function need to be maintained within the membrane of plants for the photosynthetic machinery to operate (Millar et al. 2000). In order to overcome this, plants can tolerate large amounts of unusual FAs in the storage lipids because they are sequestered into oil bodies (Millar et al. 2000). A similar experiment by Eccleston et al. (1996) showed that even though the *35S-FatB1* was inserted into *B. napus*, only significant changes occurred in the TAG fraction of the seeds. There were no changes in the leaves or roots even though higher levels of *FatB1* were present in vegetative tissue other than the seed. The ratio of $^{\Delta 9}$16:1/16:0 in leaf lipids of transformed and WT plants are depicted in Table 4 (in Appendix) and Figure 12. The $^{\Delta 9}$16:1/16:0 ratio only significantly increased in the 16-1 line, due to the decrease in 16:0 and significant increase in the $^{\Delta 9}$16:1 mole %, as seen in Figure 13. While there were only significant increases in the mole% of $^{\Delta 9}$16:1 in the *A. thaliana* leaves in line 16-1, there were significant increases in the elongated product of $^{\Delta 9}$16:1, $^{\Delta 11}$18:1, in the 16-1, 26-1, and 27-1 transformed *A. thaliana* lines. As mentioned earlier, in the investigation by Vijayan & Browse (2002), an increase in the trienoic FAs was correlated with increased chill tolerance. In the current study there was an increase, for the most part, in all trienoic FAs in the transformed plant lines compared to the WS control (Figure 13). This increase does not necessarily mean that there is an increase of trienoic FAs in the PG fraction. This aspect would need to be investigated further. Overall, the SFA content in the leaves of the transgenic *A. thaliana* remained the same or increased when compared to the untransformed WT control. This might have been caused by oxidative stress on the

plants induced by slightly higher light intensity levels, because the average light intensity during the 16h day light period was 190 μmol m^{-2} s^{-1}, compared to the more moderate level of 100 μmol m^{-2} s^{-1} reported by Rossel et al. (2002). Clearly, the FA profiles of the *A. thaliana* leaves did not alter significantly by the insertion of the cDNA encoding cat's claw desaturase with *35S* promoter. More experimentation, including low temperature treatment, is needed to determine if cat's claw 16:0-ACP desaturase leads to increased chilling tolerance in *A. thaliana*.

4.2. Seed Specific Expression of a cDNA Encoding Cat's Claw 16:0-ACP Desaturase in *B. napus*

The analysis of the transgenic *B. napus* included molecular work and analysis of the FA profile of the total acyl lipids and the acyl-CoA pool.

4.2.1 Identification of Transformation of T_1 Leaves from *B. napus* Grown in Summer 2001 using the NptII assay

Once *B. napus* T_1 plants had developed leaves, the NptII assay was carried out as an indication of the efficiency of transformation. Although the NptII assay confirms whether or not a plant is transgenic, the results only verify that the plant contains the kanamycin resistance cDNA and not necessarily the entire cDNA of interest. While the NptII assay is an indication of the transformation of the entire vector, Southern blotting is an absolute indicator of the insertion of the cDNA of interest. Analysis of inserted of cDNA encoding cat's claw 16:0-ACP desaturase can be found in section 4.2.3. Even though the cat's claw desaturase cDNA was inserted using a seed specific LFAH12

(Figure 6), the cDNA should be present in the genomic DNA throughout the plant, and therefore should be detectable. The data for the NptII assay is shown in Table 6. All plants in C_2-31A and some C_2-41 lines were positive for the kanamycin resistance cDNA, except for C_2-41-10, 11,and 12 where the results were inconclusive.

4.2.2. Identification of the Zygosity of the Kanamycin Resistance cDNA in Transgenic *B. napus* using the Embryo Assay

The embryo assay is used to determine if transgenic plants are homozygous or heterozygous for the kanamycin resistance cDNA and is determined by the ratio of green to white callus tissue formed. To do this, the embryos from developing seeds, 40 DPA, were extracted in a sterile manner and placed on the embryo assay media that contained callus forming chemicals, and also kanamycin. Each plate contained 10 embryos and two plates for each plant were dissected. The plants were grown in a growth chamber for two weeks and the ratio of green to white callus was noted. All green callus suggest the plant is homozygous for the kanamycin resistance cDNA, whereas a mixture of green and white callus implies that the plant is heterozygous for the kanamycin resistance cDNA. All white callus implies the gene is not present in the plant. An example of the embryo assay is depicted in Figure 14, with results presented in Table 7. Most plants in transformed lines C_2-31A and C_2-41 were homozygous for the kanamycin resistance cDNA. Exceptions to this included C_2-31A-8, 9, and 12 where these plants did not produce siliques even though they did germinate, and also C_2-41-1, 6, and 7 where the embryos died during the embryo assay. All the transgenic plants that produced siliques and survived during the procedure were homozygous, whereas all the non-transformed

Table 6. The results of the NptII assay on the developing seeds from *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase for plants grown in summer 2001. The + indicates that test was positive for the kanamycin gene, whereas a – implies the kanamycin gene was not present, and i indicates the result was inconclusive.

Plant	NptII	Plant	NptII	Plant	NptII
C_2-31A-1	+	C_2-41-1	+	W1	-
C_2-31A-3	+	C_2-41-2	+	W2	-
C_2-31A-4	+	C_2-41-4	+	W3	-
C_2-31A-5	+	C_2-41-6	+	W4	-
C_2-31A-6	+	C_2-41-7	+	W5	-
C_2-31A-8	+	C_2-41-8	+	W6	-
C_2-31A-9	+	C_2-41-9	+	W7	-
C_2-31A-10	+	C_2-41-10	i	W8	-
C_2-31A-11	+	C_2-41-11	i	W9	-
C_2-31A-12	+	C_2-41-12	i	W10	-
* plants C_2-31A-2 and 7, and C_2-41-3 and 5				W11	-
never germinated				W12	-

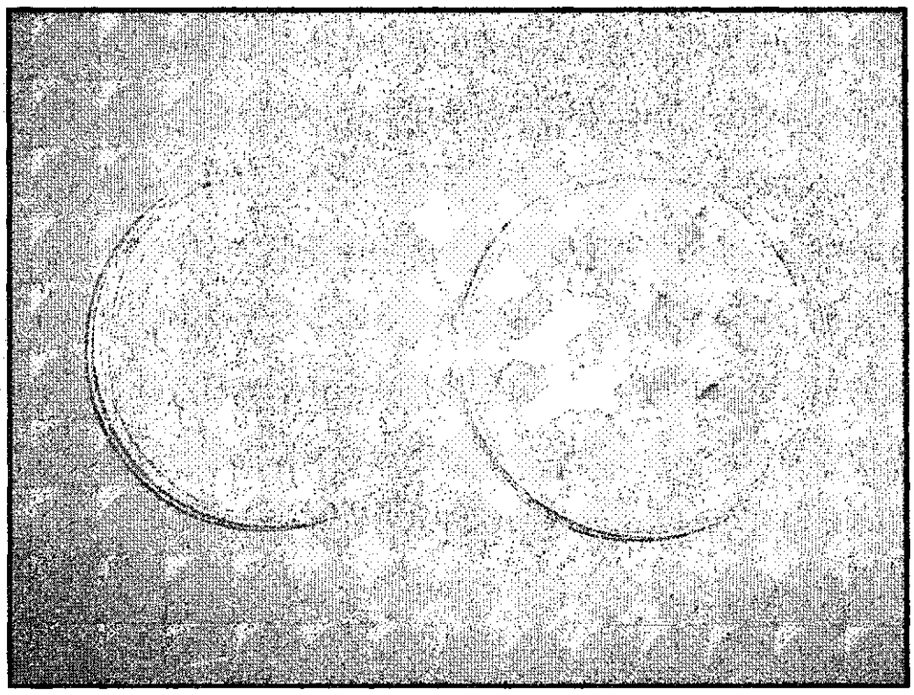

Figure 14. The callus tissue of the embryo assay in developing seeds of *B. napus* L. cv Westar and C_2-31A *B. napus*. The plate located on the left contains 10 dissected embryos from the Westar 7 plant (all white) and the plate located on the right contains 10 dissected embryos from C_2-31A-1 (all green). Both plates were grown on embryo assay media in a growth chamber with the light intensity of 70-80 mmol m^{-2} s^{-1}, on a 16 hour light/eight hour dark cycle, at 24°C, for a period of two weeks.

Table 7. Results of the embryo assay on developing seeds of *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase. Embryos were dissected in a sterile manner at around 40 DPA, but while all seeds were still green, and placed on plates containing kanamycin and also callus forming hormones. The callus were allowed to grow for two weeks, and the color and number of callus tissue was noted. The abbreviations NS means no siliques and D means died.

Plant	# Green	Plant	# Green	Plant	# Green
C_2-31A-1	20	C_2-41-1	D	W1	0
C_2-31A-3	20	C_2-41-2	20	W2	0
C_2-31A-4	20	C_2-41-4	20	W3	0
C_2-31A-5	20	C_2-41-6	D	W4	0
C_2-31A-6	20	C_2-41-7	D	W5	0
C_2-31A-8	NS	C_2-41-8	20	W6	0
C_2-31A-9	NS	C_2-41-9	20	W7	0
C_2-31A-10	20	C_2-41-10	20	W8	0
C_2-31A-11	20	C_2-41-11	20	W9	0
C_2-31A-12	NS	C_2-41-12	20	W10	0
* plants C_2-31A-2 and 7, and C_2-41-3 and 5 never germinated				W11	0
				W12	0

control Westar did not show a single green callus. This latter result was expected as the kanamycin resistance cDNA was not present within the genome of the control plants.

4.2.3. Identification of cDNA Encoding Cat's Claw 16:0-ACP Desaturase in Transgenic *B. napus* using Southern Blotting

Southern blotting was used in order to determine if the cDNA encoding cat's claw 16:0-ACP desaturase was inserted into the *B. napus* genome via probing the membrane with a cat's claw cDNA full length radiolabeled ^{32}P-dCTP, and also to determine if there were multiple copies of the exogenous cDNA. The probe was made by restricting the p566 vector, containing the entire cat's claw desaturase sequence with Sal I restriction sites on either side, with Sal I and gel cleaning the probe. See Figure 15 for a gel of the restricted p566 with Sal I, and Figure 16 for a depiction of the cleaned probe. For each plant grown in the summer of 2001, the genomic DNA was extracted and restricted with EcoRI and also PvuII, independently, as neither of these enzymes will cut within the cDNA encoding the cat's claw 16:0-ACP desaturase sequence. All of the samples were then probed twice, to determine if the copy number could be verified. Due to the large number of samples generated with EcoRI and PvuII digests, samples were run on two separate gels. The probed blots for all the membranes probed with the ^{32}P- radiolabeled cDNA encoding cat's claw 16:0-ACP desaturase for the EcoRI#1, EcoRI#2, PvuII#1, PvuII#2, are found in Figure 17, 18, 19 and 20, respectively. A summary of the number of insertions of the cat's claw cDNA can be found in Table 8.

All the transgenic *B. napus* contained more than one copy of the cDNA encoding cat's claw desaturase. There were at least two bands present in each of the transgenic

Figure 15. Ethidium bromide stained agarose gel of p566 unrestricted and restricted with SalI. The full length cDNA encoding the cat's claw 16:0-ACP desaturase was cut out of p566 plasmid with Sal I because the plasmid contained the cDNA with Sal I restriction sights on either side. The cDNA was extracted from the gel. The gel contains from left to right, in lanes (1-5) p566 uncut, with the following amounts 1) 4.2 μg, 2) 6.1 μg, 3) 3.5 μg, 4) 5.0 μg, 5) 3.8 μg and in lanes (6-10) p566 restricted with SalI, with the following amounts: 6) 4.2μg, 7) 6.1μg, 8) 3.5 μg, 9) 5.0 μg, 10) 3.8μg and 11) 8 μl λ-DNA (cut with EcoRI and HingIII) labeled in bp.

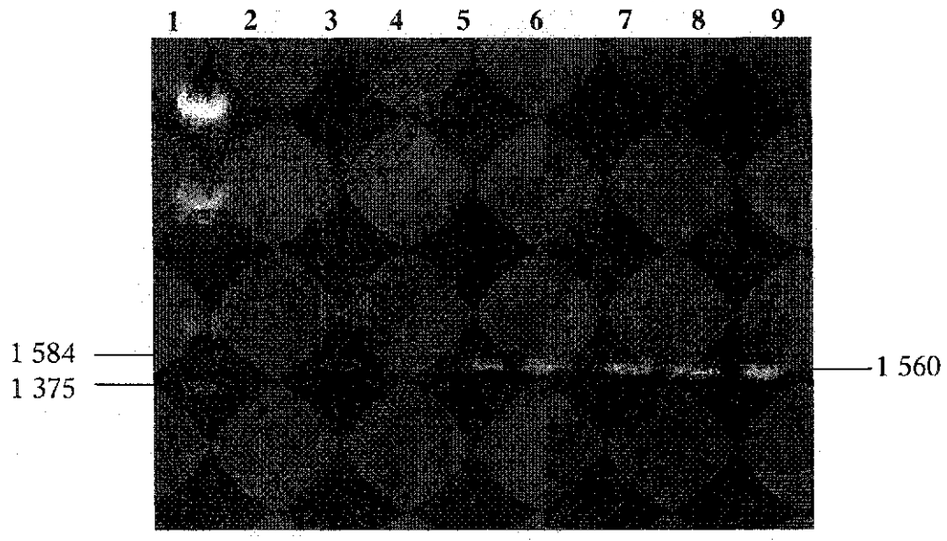

Figure 16. Ethidium bromide stained agarose gel of full-length sequence of cDNA encoding cat's claw 16:0-ACP desaturase following purification of the cDNA by electrophoresis. The 1560 bp band was extracted and gel cleaned in order to be radiolabeled for use as a probe for Southern blotting. Lanes 1 and 2 represent 8 μL λ-DNA restricted with EcoRI and HindIII and no sample, respectively. Lanes 3 - 9 represent increasing amounts of the 1560 bp cDNA encoding the cat's claw 16:0-ACP desaturase with the following amounts: 3) 0.04 μg, 4) 0.06 μg, 5) 0.08 μg, 6) 0.10 μg, 7) 0.12 μg, 8) 0.14 μg, 9) 0.16 μg.

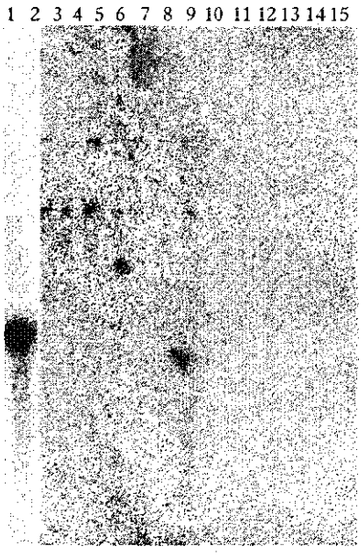

Figure 17. The Southern blot, EcoRI#1, digested genomic DNA from *B. napus* containing cDNA encoding 16:0-ACP desaturase from cat's claw. The probe was the cDNA encoding cat's claw desaturase (1560 bp) labeled with ^{32}P-dCTP. The contents of the lanes are as follows lane 1) 0.1μg positive control (1560 bp cats claw desaturase sequence), 2) C_2-31A-1, 3) C_2-31A-3, 4) C_2-31A-5, 5) C_2-41-1, 6) C_2-41-2, 7) C_2-41A-4, 8) C_2-41A-6, 9) C_2-41-7, 10) Westar 1, 11) Westar 2, 12) Westar 3, 13) Westar 4, 14) Westar 5, and 15) Westar 6. All *B. napus* samples contain 20 μg of genomic DNA.

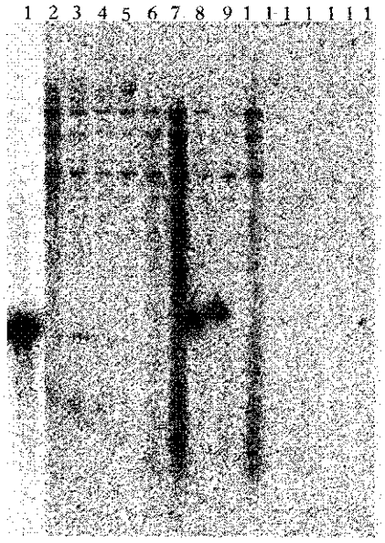

Figure 18. The Southern blot, EcoRI*2, digested genomic DNA from *B. napus* containing cDNA encoding 16:0-ACP desaturase from cat's claw. The probe used was the cDNA encoding the cat's claw desaturase (1560 bp) labeled with ^{32}P-dCTP. The contents of the lanes are as follows: 1) positive control (1560 bp cats claw desaturase sequence), 2) C_2-31A-6, 3) C_2-31A-10, 4) C_2-31A-11, 5) C_2-31A-12, 6) C_2-41-8, 7) C_2-41-9, 8) C_2-41-10, 9) C_2-41-11, 10) C_2-41-12, 11) Westar 7, 12) Westar 8, 13) Westar 9, 14) Westar 10, 15) Westar 11, and 16) Westar 12. All *B. napus* samples contain 20 μg of genomic DNA.

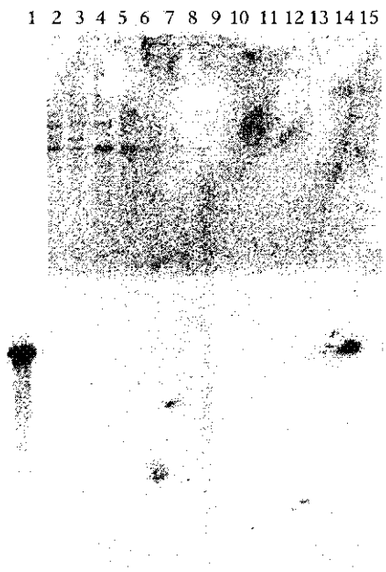

Figure 19. The Southern blot, PvuII[#]1, digested genomic DNA from *B. napus* containing cDNA encoding 16:0-ACP desaturase from cat's claw. The probe was the cDNA encoding cat's claw desaturase (1560 bp) labeled with ^{32}P-dCTP. The contents of the lanes are as follows: 1) positive control (1560 bp cats claw desaturase sequence), 2) C_2-31A-1, 3) C_2-31A-3, 4) C_2-31A-5, 5) C_2-41-1, 6) C_2-41-2, 7) C_2-41A-4, 8) C_2-41A-6, 9) C_2-41-7, 10) Westar 1, 11) Westar 2, 12) Westar 3, 13) Westar 4, and 15) Westar 5. All *B. napus* samples contain 20 μg of genomic DNA.

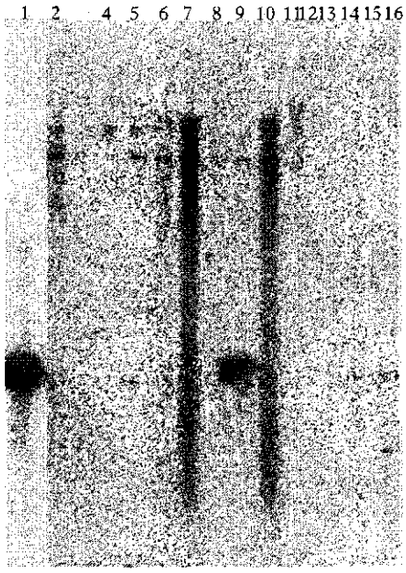

Figure 20. The Southern blot, PvuII#2, digested genomic DNA from *B. napus* containing cDNA encoding 16:0-ACP desaturase from cat's claw. The probe was the cDNA encoding cat's claw desaturase (1560 bp) labeled with ^{32}P-dCTP. The contents of the lanes are as follows: 1) positive control (1560 bp cats claw desaturase sequence), 2) C_2-31A-6, 3) C_2-31A-10, 4) C_2-31A-11, 5) C_2-31A-12, 6) C_2-41-8, 7) C_2-41-9, 8) C_2-41-10, 9) C_2-41-11, 10) C_2-41-12, 11) Westar 7, 12) Westar 8, 13) Westar 9, 14) Westar 10, 15) Westar 11, and 16) Westar 12. All *B. napus* samples contain 20 μg of genomic DNA.

Table 8. Southern blotting of *B. napus* to determine if the cDNA encoding cat's claw 16:0-ACP desaturase was present in the genome. Genomic DNA of freeze dried leaf tissue samples from transformed plants was restricted with EcoRI and PvuII, independently, and the membrane was probed with the ^{32}PdCTP labeled full-length cat's claw cDNA encoding 16:0-ACP desaturase. The number of copies of the gene found from the Southern blot are indicated for each plant tested.

Plant Sample	Number of Gene Copies		Plant Sample	Number of Gene Copies		Plant Sample	Number of Gene Copies	
	EcoRI	PvuII		EcoRI	PvuII		EcoRI	PvuII
C_2-31A-1	3	3	C_2-41-1	3	2	Westar 1	0	0
C_2-31A-3	3	3	C_2-41-2	3	2	Westar 2	0	0
C_2-31A-5	3	2	C_2-41-4	3	1	Westar 3	0	0
C_2-31A-6	6	4	C_2-41-6	3	1	Westar 4	0	0
C_2-31A-10	6	2	C_2-41-7	3	1	Westar 5	0	0
C_2-31A-11	5	2	C_2-41-8	4	2	Westar 6	1	0
C_2-31A-12	6	2	C_2-41-9	4	2	Westar 7	1	0
			C_2-41-10	4	2	Westar 8	1	0
			C_2-41-11	4	2	Westar 9	1	0
			C_2-41-12	4	2	Westar 10	1	0
						Westar 11	1	0
						Westar 12	1	0

plant samples tested (Table 8). Transformed plants can contain more than one copy of the inserted gene as a single bacterium can transfer more than one copy of the T-DNA during the transfer, which could enter the plant genome randomly (Walden 1988). The number of copies could only be verified for C_2-31A-1 and C_2-31A-3 because there were three copies of the desaturase cDNA in the case of either restriction enzyme. In the case of digestion with EcoRI, there were more copies on average per plant when compared to digests with PvuII, and would be due the fact that EcoRI obviously cuts more often within the *B. napus* genome than PvuII.

In the case of the Westar control plants, including plants 6 through 12, there was hybridization for the desaturase cDNA. This is found on the EcoRI#2 blot (Figure 18), and also aligned with a band that is present in all the transformed *B. napus* plants and is likely due to hybridization of probe to the gene encoding endogenous $^{\Delta 9}$18:0-ACP desaturase. This degree of hybridization can be explained based on the high sequence homology between the cDNA encoding cat's claw desaturase and the endogenous $^{\Delta 9}$18:0-ACP desaturase because the DNA sequences were found to have a 75% sequence homology when a two sequence BLAST comparison was performed (data not shown). Although these bands were present faintly in the case of the EcoRI#2 blot (Figure 18), these inserts were not found on the PvuII#2 blot (Figure 19) or any other Westar sample on the EcoRI#1 or PvuII#1 blots (Figures 17 and 19, respectively). There are no PvuII restriction site within the coding $^{\Delta 9}$18:0-ACP desaturase and as long as there are also none present within the introns of the endogenous $^{\Delta 9}$18:0-ACP desaturase, PvuII would not cut the 18:0-ACP desaturase and result in a loss of hybridization to the probe. Most likely this band does not show up because there is much less hybridization and therefore

the intensity is much less when compared to the hybridization of the cDNA encoding the 16:0-ACP desaturase sequence.

Lastly, there is evidence that the plants that were undistinguishable in the NptII assay were, in fact, transformed and include C_2-41-10, C_2-41-11, C_2-41-12. All of these plants were positive for more than one cDNA encoding cat's claw 16:0-ACP desaturase gene.

4.2.4. Analysis of Transcripts Encoding Cat's Claw 16:0-ACP Desaturase using RT-PCR and Southern Blotting

Transcript analysis was conducted as a follow up to detecting the cDNA encoding cat's claw 16:0-ACP desaturase in the *B. napus* genome. Due to the lack of RNA present, RT-PCR was performed using random primers and then Southern blotting techniques were used. The bands present on the agarose gel of the RT-PCR products were very faint, see gel in Figure 21. The RT portion was done using random primers and a 38-cycle PCR program for the primers was made. Thirty-eight cycles were chosen because this was the highest number of cycles that could be accommodated while still in the linear range, with the equation of the line of $y = 1.645x - 47.75$, R^2 0.979 (data not shown). Once the gel was transferred to the nylon membrane, the membrane was probed twice. The initial probe was the ^{32}P-dATP-labeled RT-PCR product made by primers designed to amplify a 760 bp sequence within the cDNA encoding the cat's claw 16:0-ACP desaturase sequence, (see Figure 8 for forward and reverse primers and the primed sequence). The second ^{32}P-dATP-labeled RT-PCR probe used was made from amplifying mitochondrial elongation factor Tu (EFTu) using primers to generate a 330 bp

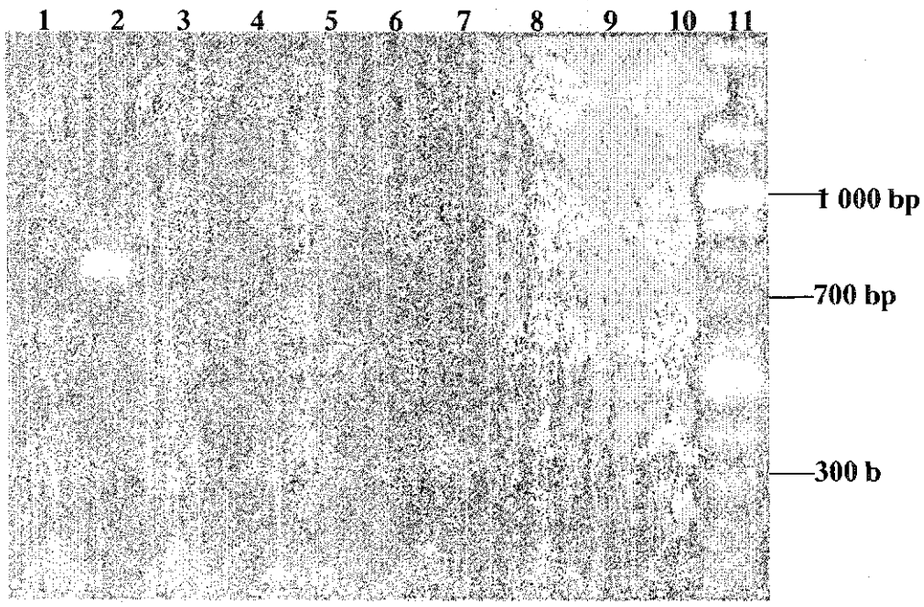

Figure 21. Ethidium bromide staining of electrophoresis resolved RT-PCR products from amplification of the 760 bp cDNA encoding 16:0-ACP cat's claw destaruase and 330 bp EFTu. Contents of lanes: 1) negative control containing no desaturase template, 2) positive control for the 760 bp cats claw desaturase gene, 3) C_2-31A-1, 4) C_2-31A-10, 5) C_2-41-1, 6) C_2-41-6, 7) C_2-41-10, 8) C_2-41-11, 9) Westar 3, 10) Westar 6 and 11) 2.5 μg 100 bp ladder. All *B. napus* samples started with 60ng of RNA in the RT reaction, with 4uL of RT product was used for PCR, and 45uL of the total 55 uL sample loaded (50uL RT-PCR product and 5 uL of 6x loading dye) on an agarose gel.

cDNA as a control. The EFTu was chosen as a control as a previous study demonstrated that the cDNA for EFTu appeared to be constant (Weselake et al. 1998). Electrophoresis purified 760 bp cDNA encoding 16:0-ACP desaturase and the 330 bp cDNA encoding EFTu can be seen in Figure 22. Results for the membrane probed initially with ^{32}P-dATP-labeled 760 bp desaturase is shown in Figure 23, and results for the membrane probed with both^{32}P-dATP labeled 760 bp 16:0-ACP desaturase RT-PCR product and the ^{32}P-dATP-labeled EFTu RT-PCR product are shown in Figure 24. The 760 bp cDNA from cat's claw encoding 16:0-ACP desaturase appears to be within the *B. napus* genome in the C_2-41-6 and C_2-41-11 (Figure 23). These samples, along with samples C_2-31A-1 and C_2-31A-10, have the cDNA encoding EFTu at 330 bp (Figure 24). While the bands corresponding to the cDNA encoding EFTu in C_2-31A-1 and C_2-31A-10 and C_2-41-6 are about the same intensity, sample C_2-41-11 has a slightly lighter 330 bp EFTu band. Thus, there was an increase in the amount of transcription of the cDNA encoding cat's claw 16:0-ACP desaturase in sample C_2-41-11 when compared to C_2-41-6. This was due to a greater amount of the RNA used in the initial RT portion of the experiment, and can be seen in the more intense band corresponding to the cDNA encoding EFTu (330 bp) in C_2-41-6 compared to C_2-41-11. Assuming that these results were representative of the entire population of plants grown, one would expect an increase in the transcription of the cDNA encoding cat's claw 16:0-ACP desaturase in the C_2-41 transformed line compared to the C_2-31A line. While the cDNA encoding EFTu (330 bp) was present in C_2-31A-1, C_2-31A-10, C_2-41-6 and C_2-41-11, there was no band present in Westar 3 and Westar 6. One would expect to see a band for the cDNA encoding EFTu in each Westar 3 and 6, as the EFTu should be found in transformed and non-transformed plants alike. The cDNA

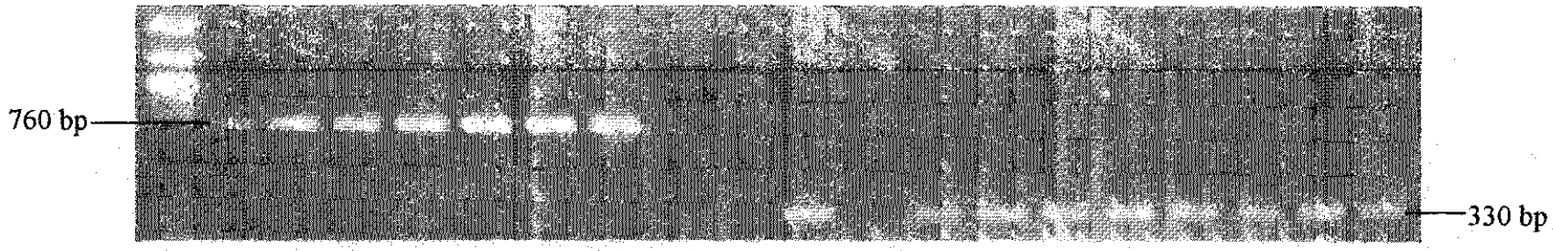

Figure 22. Ethidium bromide staining of the resolved RT-PCR products from the amplification the 760 bp in the cDNA encoding cat's claw 16:0-ACP desaturase and the 330 bp nucleic acid sequence for EFTu in order to be used for radiolabeled probes. The cat's claw 16:0-ACP desaturase probe was designed using primers to amplify a 760 bp, a partial sequence, within the cDNA encoding cat's claw 16:0-ACP desaturase sequence and is present on the left hand side of the gel. The 330 bp probe was made using the EFTu nucleic acid primers to amplifying EFTu cDNA. The PCR product of a Westar control plant and can be seen on the right side of the gel, and the DNA nucleic acid markers on the far left is the 100 bp ladder.

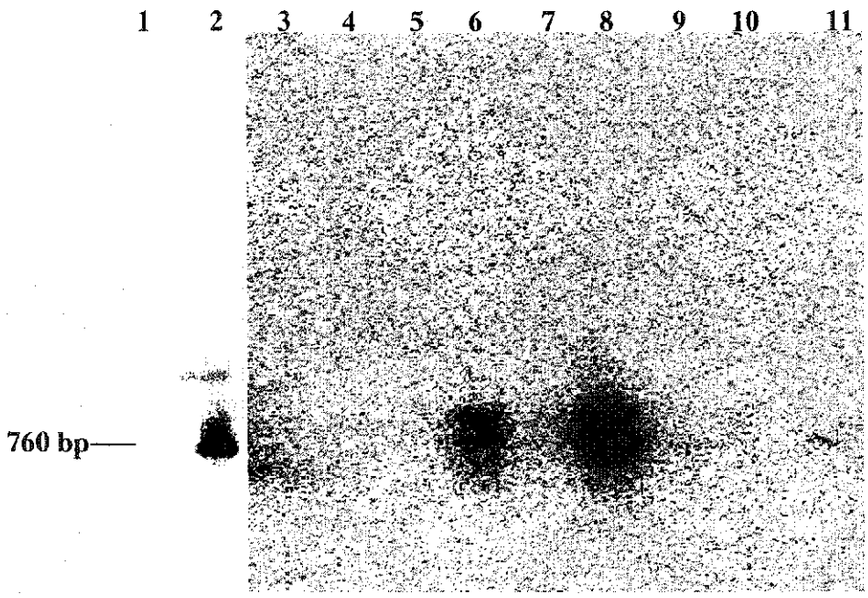

Figure 23. Southern blotting of the RT-PCR products from the amplification of the 760 bp cDNA encoding cat's claw 16:0-ACP desaturase and the 330bp EFTu using ^{32}P-dATP labelled 760 bp cDNA encoding the cat's claw desaturase. Contents of lanes: 1) negative control contaning no template, 2) positive control for the 760 bp cats claw desaturase gene, 3) C_2-31A-1, 4) C_2-31A-10, 5) C_2-41-1, 6) C_2-41-6, 7) C_2-41-10, 8) C_2-41-11, 9) Westar 3, 10) Westar 6 and 11) 2.5 μg 100 bp ladder. All *B. napus* samples started with 60ng of RNA in the RT reaction mixture, with 4uL of RT product was used for PCR, and 45uL of the total 55 uL sample loaded (50uL RT-PCR product and 5 uL of 6x loading dye) on the agarose gel.

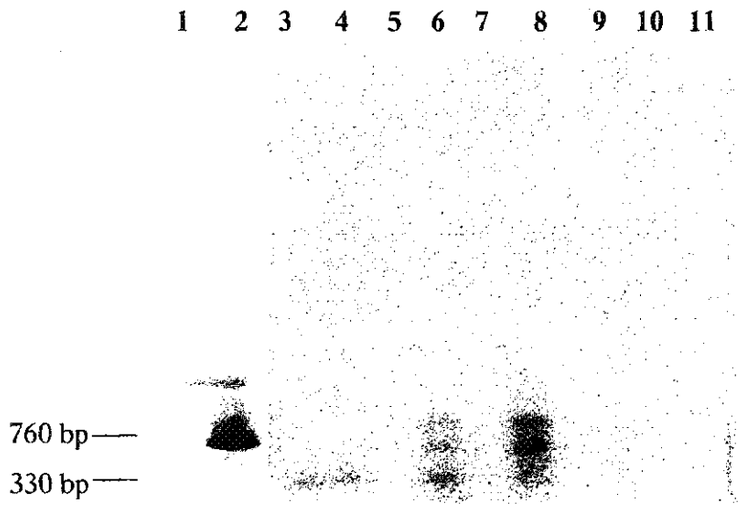

Figure 24. Southern blotting of the RT-PCR products from the amplification of the 760 bp cat's claw 16:0-ACP desaturase and 330 bp from the EFTu using radio - labeled ^{32}P-dATPs including both the cDNA encoding 760 bp cat's claw 16:0-ACP desaturase partial sequence and the 330 bp nucleic acid sequence from EFTu. Contents of lanes: lane 1) negative control containing no template, 2) positive control for the 760 bp cats claw desaturase gene, 3) C_2-31A-1, 4) C_2-31A-10, 5) C_2-41-1, 6) C_2-41-6, 7) C_2-41-10, 8) C_2-41-11, 9) Westar 3, 10) Westar 6 and 11) 2.5 μg 100 bp ladder. All *B. napus* samples started with 60 ng of RNA in the RT reaction mixture, with 4uL of RT product used for PCR, and 45uL of the total 55 uL sample loaded (50uL RT-PCR product and 5 uL of 6x loading dye) on the agarose gel.

probe encoding EFTu was made by the PCR amplification of untransformed Westar plants and should have the same sequence as the Westar plants. The lack of a band in both Westar plants indicates the RNA used may not have been equivalent in each sample or the RNA was degraded.

While these results are an indication that the introduced cDNA was being transcribed, it cannot be assumed that $^{\Delta 9}$16:0-ACP cat's claw desaturase protein was being synthesized. Polyclonal antibodies were raised against a stretch of amino acid sequence in the signal peptide to $^{\Delta 9}$16:0-ACP cat's claw desaturase that was different from the amino acid sequences of the $^{\Delta 9}$18:0-ACP from *B. napus*. The antibodies, however, were not sufficiently specific to assess 16:0-ACP desaturase synthesis in developing seeds from transgenic plants.

4.2.5. The Effects on the FA Profile of Neutral Lipids in *B. napus* Transformed with the cDNA Encoding Cat's Claw 16:0-ACP Desaturase and Grown in Summer 2001

The neutral lipids from the *B. napus* grown in the summer 2001 were extracted using PE extraction and the NaMeth methylation of FAs (Table 9 in Appendix and Figure 25). Other extraction and methylations methods were tried and results for these methods are shown in Tables 12-15 (in Appendix) and Figures 36 –39 (in Appendix). There were significant increases in the SFA content of both transformed lines grown, which resulted from an increase in almost all the SFAs present. The increase in the amount of SFA may have been a consequence of high greenhouse temperatures during the summer growing season. While the exact temperatures are not known, often the temperature was around

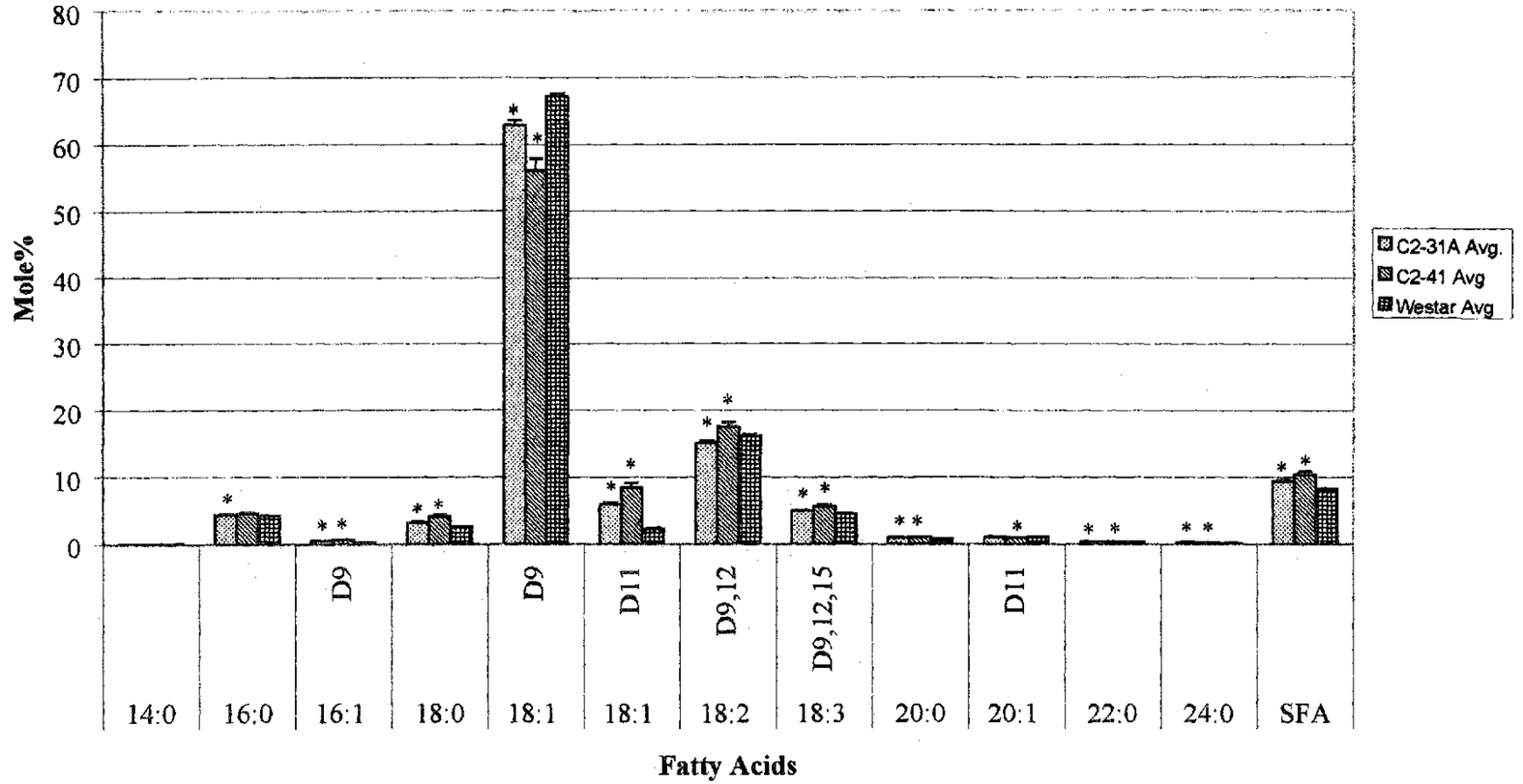

Figure 25. FA composition of seed total acyl lipids from *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using the PE-extracted lipid and NaMeth derivitized FAs- summer 2001 plants. The two transformed lines C_2-31A (n=7) and C_2-41 (n=10) were compared to Westar (n=12) using ANOVA testing and all significant differences are indicated with a *.

37°C. Although there was an increase in the amount of SFAs, there were also other changes in the FA profile. These included a significant decrease in the 16:0 content of the C_2-31A transformed line, an increase in the amount of $^{\Delta 9}$16:1 and the elongation product $^{\Delta 11}$18:1. As well the $^{\Delta 9}$16:1/16:0 ratio increased significantly for both transformed lines compared to the control, see Figure 26 for comparison of the ratios. There were also many other significant changes in the FA profile compared to the control, see Figure 25, for these changes.

In summary, although there was an increase in the amount of SFA, the 16:0-ACP desaturase appeared to be functioning within the transformed *B. napus*. The larger $^{\Delta 9}$16:1/16:0 ratio for the C_2-41 line compared to the C_2-31A line suggests that the cat's claw desaturase was more active within the C_2-41 transformed line.

4.2.6. The Effects on the FA Profile of Neutral Lipids in *B. napus* Transformed with Cat's Claw 16:0-ACP Desaturase and Grown in Fall 2001

The FA profile from the plants grown in the fall of 2001 can be seen in Table 10 (in Appendix) and Figure 27. Here, there were no significant changes in the amount of SFA present in the transformed lines compared to the untransformed Westar control. There were, however, significant increases in the amount of $^{\Delta 9}$16:1 and the elongation product $^{\Delta 11}$18:1, as was the case in the *B. napus* grown in summer. There was also a significant decrease in the amount of 16:0 present in the C_2-41 line. These changes indicated that the cat's claw 16:0-ACP desaturase was active within *B. napus*. There were also other significant changes within the FA profile, including decreases in the amount $^{\Delta 9}$18:1 and other FAs (Figure 27). While there was no change in the SFA

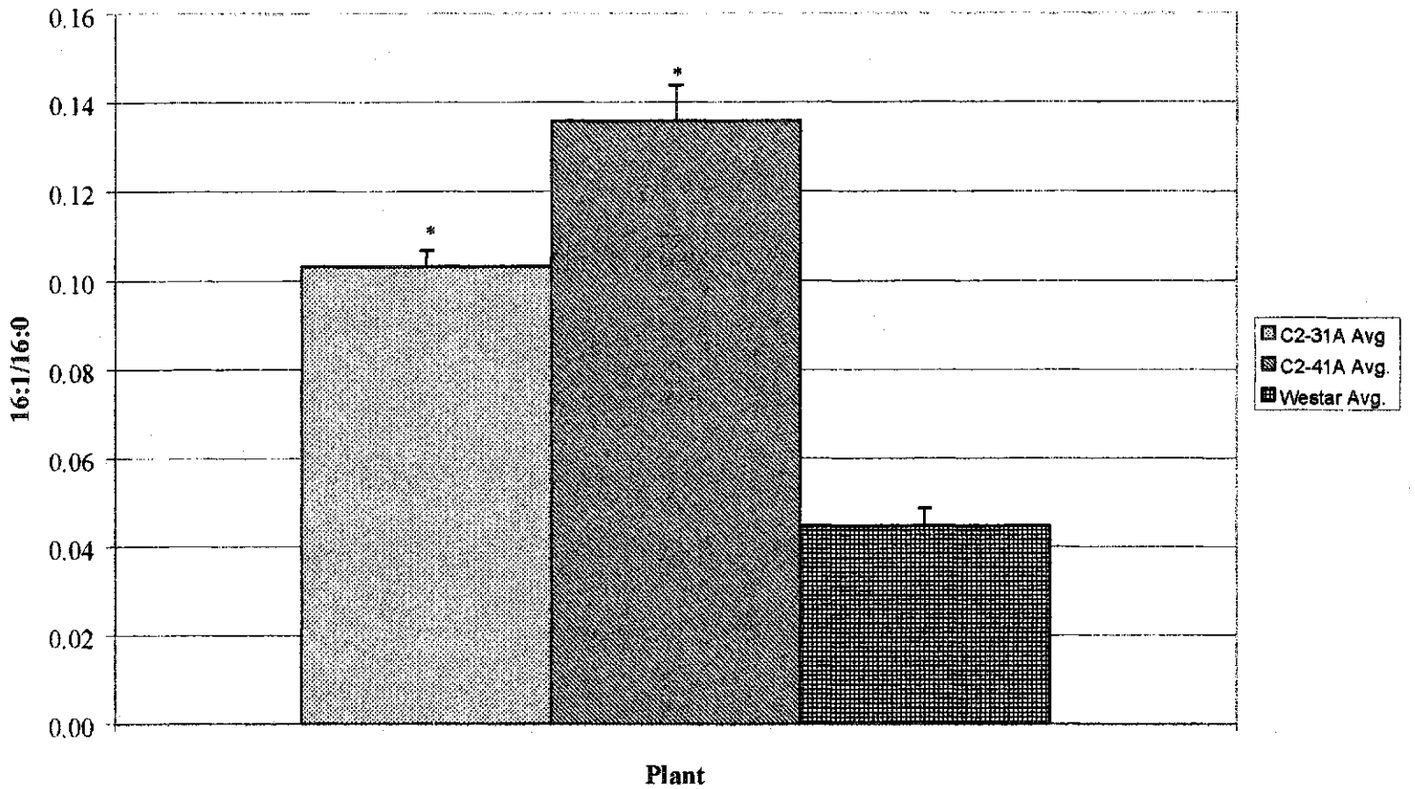

Figure 26. 16:1/16:0 ratio of seed oil from *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase - summer 2001 plants. Total lipids were extracted using PE extraction and NaMeth methlylation. Plants included C_2-31A (n=7), C_2-41 (n=10), and Westar (n=12). ANOVA testing was performed and all significant differences are shown with a *.

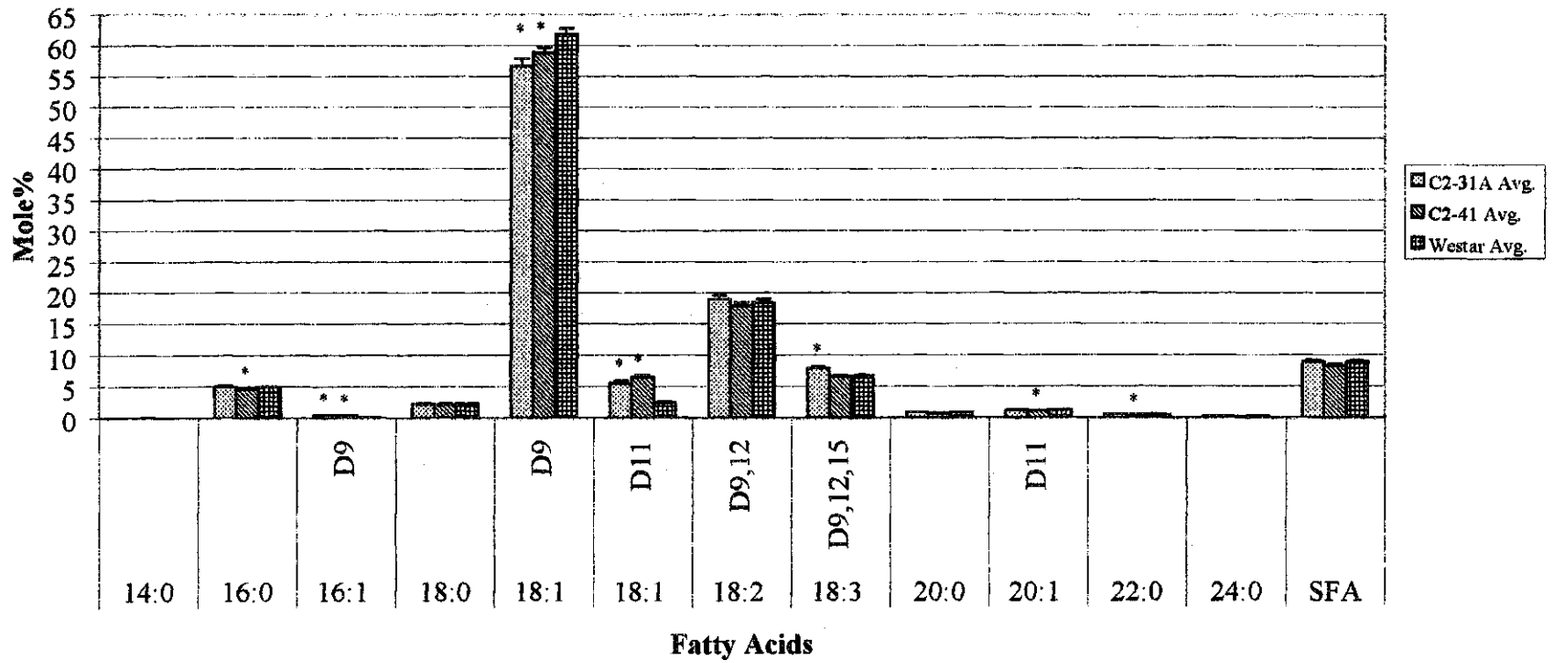

Figure 27. The FA composition (mole%) of *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase-fall 2001 plants. The total lipids were extracted with PE and methylated with NaMeth. The transformed lines C_2-31A (n=12) and C_2-41 (n=15) were compared to the untransformed control, Westar (n=15) using ANOVA testing and all significant differences are shown with *.

content, there were significant increases in the $^{\Delta 9}$16:1/16:0 ratios for both transformed plant lines compared to the control (Figure 28). Again, the higher $^{\Delta 9}$16:1/16:0 ratio can be seen in the C_2-41 line suggesting that there was a greater amount of $^{\Delta 9}$16:0-ACP desaturase activity in this transformed line compared to the C_2-31A line.

In summary, the insertion of the cDNA encoding cat's claw 16:0-ACP desaturase caused many changes within *B. napus*, including significant increases in the mole% of $^{\Delta 9}$16:1 and its elongated product $^{\Delta 11}$18:1. While the insertion of the cDNA encoding cat's claw 16:0-ACP desaturase did increase the expected FAs, it did not cause an overall change in the total amount of SFA content in the transformed lines. This may be explained due to the reduced selectivity of endogenous ATs towards the $^{\Delta 9}$16:1.

Chung Suh et al. (2002) investigated the effects of the introduction of three different acyl-ACP desaturases into *A. thaliana* on the FA profile of the seed. The desaturases were all expressed using seed specific napin promoters and included a cDNA encoding $^{\Delta 4}$16:0-ACP desaturase product from coriander, a $^{\Delta 6}$16:0-ACP desaturase from *T. alata* and a $^{\Delta 9}$14:0-ACP desaturase from geranium. In the plants expressing the $^{\Delta 4}$16:0-ACP coriander desaturase, the seed's oil contained $^{\Delta 4}$16:1 and $^{\Delta 6}$18:1 ranging from 0.5 – 1% of the seeds FA, while in *T. alata* there was no $^{\Delta 4}$16:1 detected, but the elongation products $^{\Delta 8}$18:1 and $^{\Delta 10}$20:1 were produced at a combined level of about 4%. Similar results were found in the case of the *A. thaliana* that contained a $^{\Delta 9}$14:0-ACP desaturase, as there was no $^{\Delta 9}$14:1 detected the elongation products $^{\Delta 11}$16:1, $^{\Delta 13}$18:1 and $^{\Delta 15}$20:1 were present. These FAs ranged from trace amounts to 13%, with most lines containing nearly 7%. These results showed that the products of these three different unusual acyl-ACP desaturases remain a minor component in the seed oil. To investigate whether the

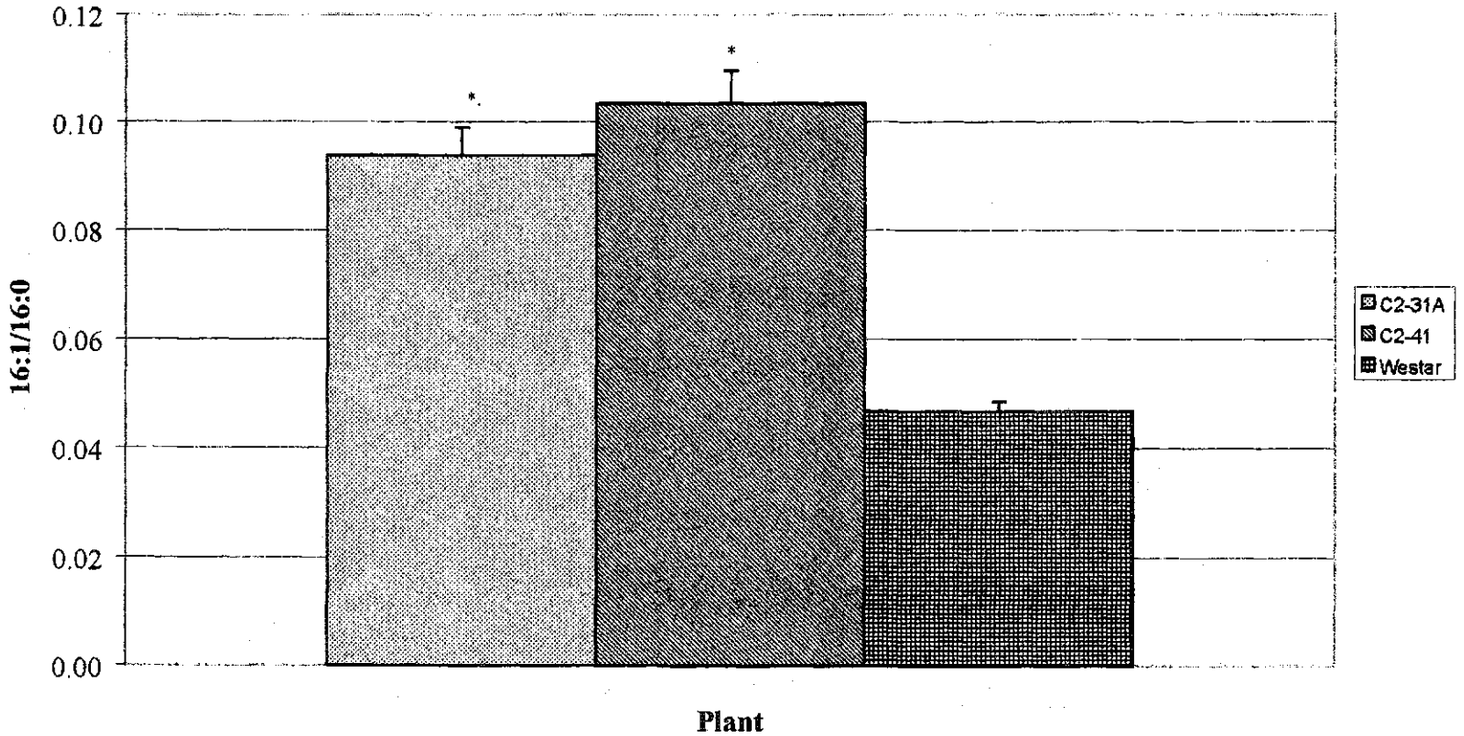

Figure 28. 16:1/16:0 ratio of seed oil from *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase-fall 2001 plants. Transformed plant lines include C_2-31A (n=12) and C_2-41 (n=15) and were compared to the untransformed control Westar (n=15) using ANOVA testing, all significant differences are shown with a *.

monoene production was limited by low desaturase expression, the levels of the $^{\Delta 4}$16:0-ACP desaturase and $^{\Delta 6}$16:0-ACP desaturase in transgenic plants were estimated by Western blotting and compared to the endogenous $^{\Delta 9}$18:0-ACP desaturase. The levels of both were at least equal to that of the endogenous $^{\Delta 9}$18:0-ACP desaturase and in some cases even higher. This result clearly showed that the accumulation of the unusual FAs did not correlate with the desaturase expression. Also, loss of unusual FAs due to β–oxidation was eliminated as a possibility for limited production of the unusual FAs because the acyl-CoA oxidase activity did not increase. Lack of substrate was also eliminated using *fab1* mutants. The *fab1* mutants have an abundance of 16:0 substrate because *fab1* controls the elongation of 16:0 to 18:0. Here the *fab1* mutants were also transformed with $^{\Delta 4}$16:0-ACP desaturase and $^{\Delta 6}$16:0-ACP desaturase and the FA profile of the transformed plants were investigated. The WT contained < 1% of $^{\Delta 4}$16:1 and $^{\Delta 6}$18:1, while the *fab1* mutant had nearly 3%. The ratio of the $^{\Delta 4}$16:1/$^{\Delta 6}$18:1 (2:1) were similar in both, leading to the conclusion that the increase in the monoenes relative to the WT was not increased. The investigation also reported low activity for the unusual acyl-ACP desaturases in transgenic lines $^{\Delta 6}$16:0 and $^{\Delta 9}$14:0 compared to the endogenous $^{\Delta 9}$18:0-ACP desaturase activity, and were thought to be due to the proper forms of ACP and Fd needed for optimal enzyme activity. The investigators also eliminated lack of proper ATs. Lastly, they determined the incorporation of [H^3] $^{\Delta 6}$18:1 and [C^{14}] $^{\Delta 9}$18:1 uptake into the TAG. This seems rather presumptive considering that 18:1 is a major FA in *A. thaliana* and therefore may include $^{\Delta 6}$18:1 simply due to its chain length and not the exact position of the double bond. While all of these suggestions are ideas to the limitation of unusual FA present in the oil of transformed plants, there is evidence that

the ATs selectivity is responsible for limited amounts of $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1. This aspect will be discussed further in section 4.2.9.

4.2.7. Seeds Oil Content Determination by LR-NMR of *B. napus* Transformed with a cDNA Encoding Cat's Claw 16:0-ACP Desaturase - Fall 2001 Plants

If the FA composition of an oil seed is altered through genetic engineering, it is also important that oil yield is not affected in order to justify the value of the transformation. To determine the percentage of oil present in *B. napus* transformed with the cDNA encoding cat's claw 16:0-ACP desaturase compared to control Westar LR-NMR was used (Figure 29). Results showed a significant decrease in the amount of oil present in the C_2-31A transformed line, whereas there was a slight increase in the amount of oil in the C_2-41 line. The reduction in oil content of line C_2-31A may be due to the stress placed on the plant because of the transformation process, or the random insertion of the cDNA encoding cat's claw 16:0-ACP desaturase into the genome may have disrupted a gene associated with oil formation.

4.2.8. Comparison of the FA Composition of Seed Oil from the *B. napus* Grown in the Summer and Fall of 2001

The differences within the FA profiles from the *B. napus* grown in the summer and the fall 2001 were compared using ANOVA testing. The differences in the profiles can give insight on the effect of increased temperature on the transformed plants. As stated earlier, the exact temperature was not recorded for the *B. napus* grown in the summer, but the temperature was often as high as 37°C, whereas the *B. napus* grown in

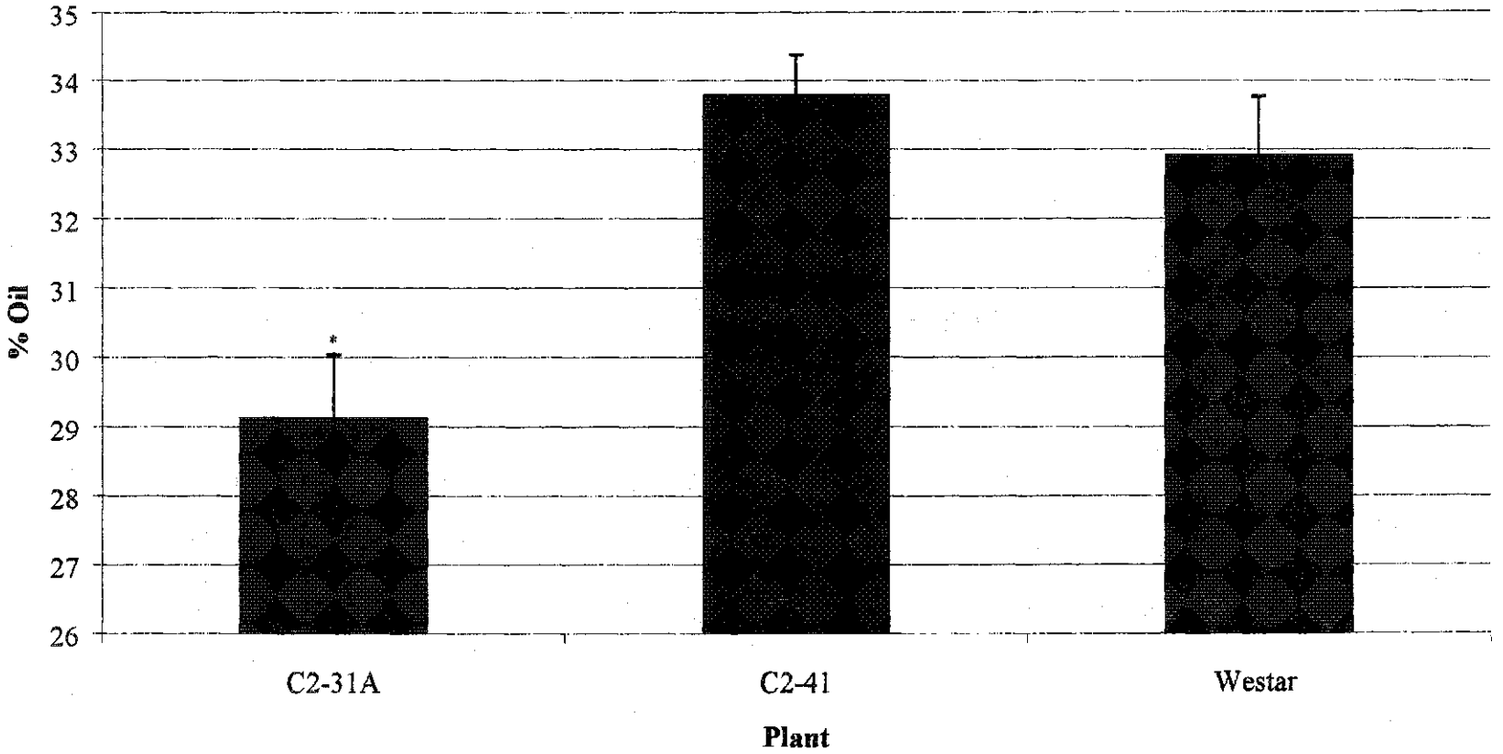

Figure 29. The average % oil (w/w) in *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using LR-NMR-fall 2001 plants. The transformed lines C_2-31A (n=12) and C_2-41 (n=14) are compared to untransformed Westar control (n=13) using ANOVA, and all significant differences are shown with *.

the fall had the average temperature high of 31.34 ± 4.30°C and the average temperature low of 15.35 ± 3.28 °C. Comparison of the plant lines grown in the summer to those grown in the fall showed significant differences in the FA profiles (Table 11 in Appendix and Figure 30).

B. napus grown in the summer had a much higher SFA content in the transgenic plant lines than the untransformed Westar control (Figure 31), and was probably a result of the unstable and newly transformed plant lines. The SFAs following this trend included 20:0 and 24:0, while all plant lines had significant increases in 18:0.

When investigating the effect of the transformation with a cDNA encoding cat's claw 16:0-ACP desaturase on the plant lines grown in the summer and the fall, striking differences were seen. While all plant lines had reduced amounts of 16:0 in the summer compared to the fall, the amount of $^{\Delta 9}$16:1 (for C_2-41 only) and $^{\Delta 11}$18:1 were much higher in the transformed lines, suggesting that the desaturase activity was also higher at the warmer growing temperatures. Both increases in SFA and increase in desaturase at warmer temperatures may be a result of stress on the newly transformed plants. The $^{\Delta 9}$16:1/16:0 ratios were compared between growing seasons and the only significant difference arose in the plant line C_2-41 (Figure 32). As demonstrated earlier in the thesis, there were more cat's claw desaturase cDNA inserts in C_2-41 compared to C_2-31A, and while there seemed to be more desaturation of 16:0 in this plant line, this plant line was also the most affected by elevated temperature. There was significantly more $^{\Delta 9}$18:1 present at the higher temperatures in C_2-31A and the Westar control, whereas there was less of this FA at the warmer temperature in C_2-41. Other changes included reductions

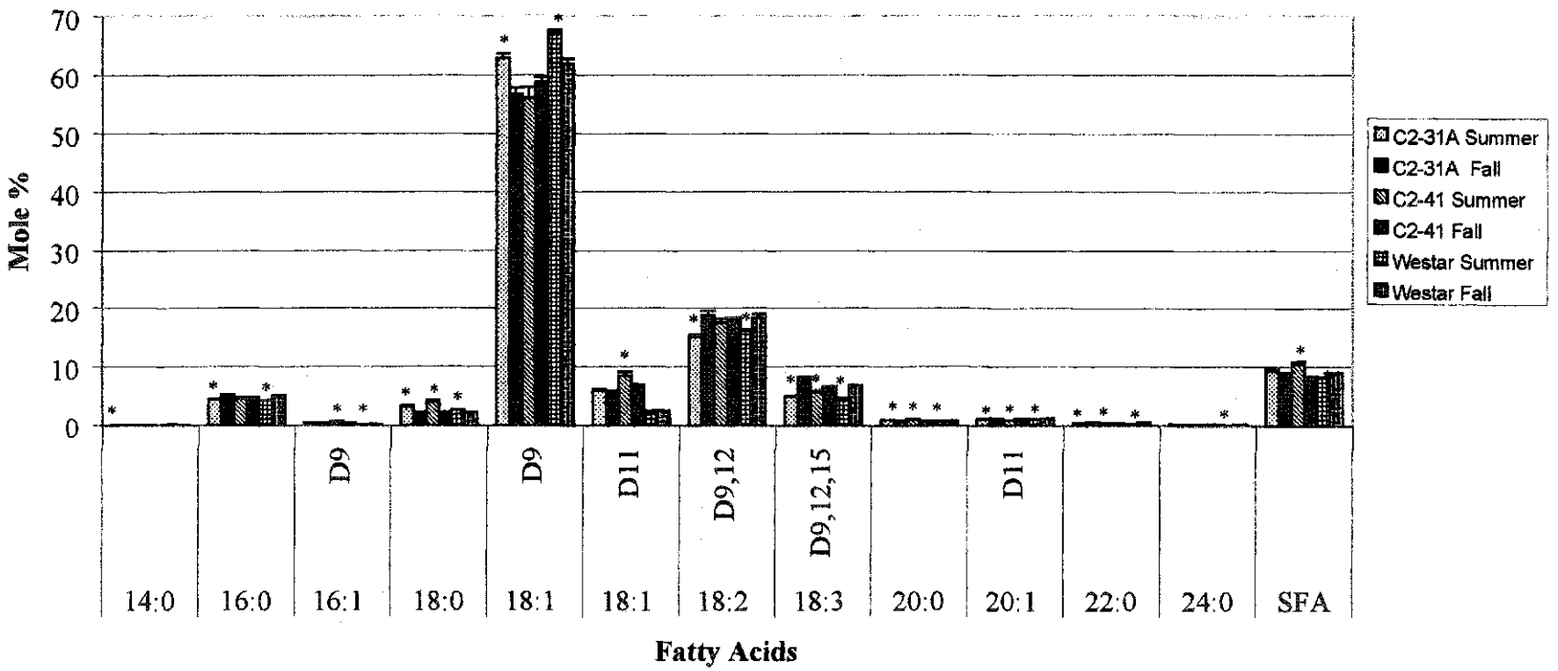

Figure 30. Comparison of FAs in *B. napus* transformed with a cDNA encoding the cat's claw 16:0-ACP desaturase- summer and fall 2001 plants. ANOVA testing was performed comparing the transformed line C_2-31A grown in summer (n=7), and C_2-31A grown in fall (n=12) this was also performed between C_2-41 grown in summer (n=10), and C_2-41 grown in fall (n=15), and Westar grown in summer (n=12) and in Westar grown in fall (n=15) and all significant differences are shown with a *.

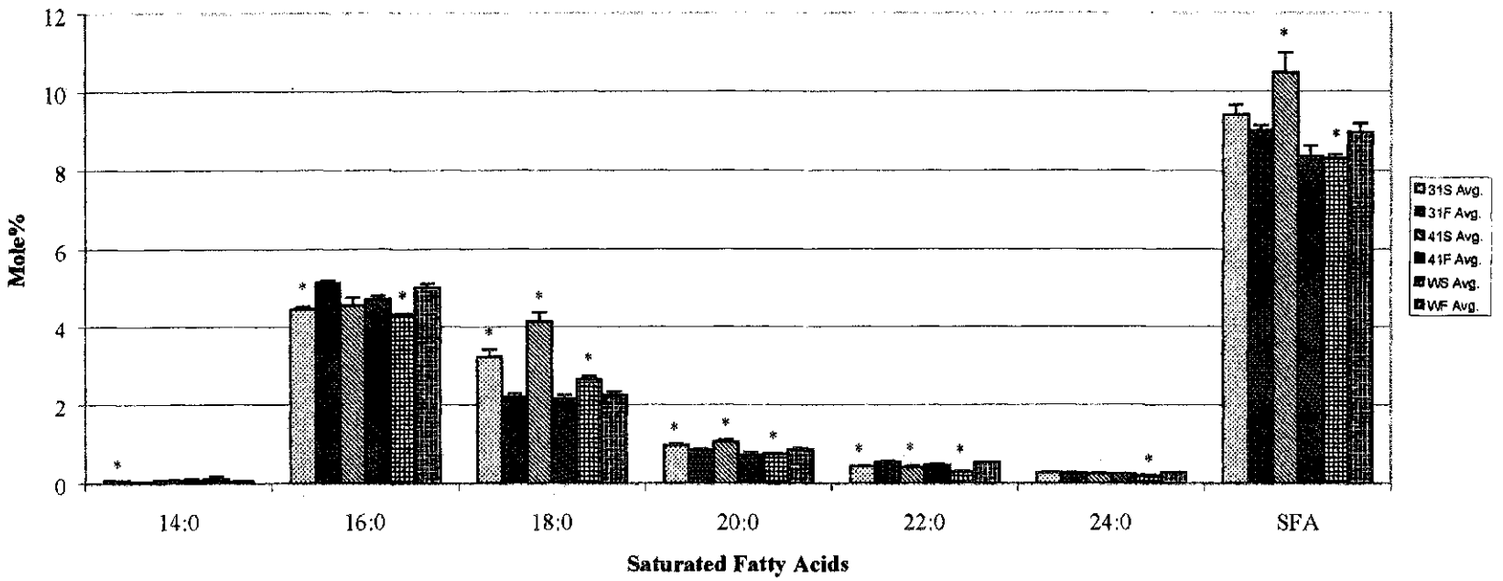

Figure 31. Comparison of SFA (mole%) in control and *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase-summer and fall 2001 plants. Plants were grown under different temperatures and due to poor ventilation in the summer, the temperature in the greenhouse often reached 37°C. In the fall, the average high was 31.34 ± 4.30°C and the average low was 15.35 ± 3.28°C. The plants lines compared using ANOVA testing and included C_2-31A Summer (n=7) and C_2-31A Fall (n=12), C_2-41 Summer (n=10) and C_2-41 Fall (n=15) and lastly Westar Summer (n=12) and Westar Fall (n=15). All significant differences are shown with a *.

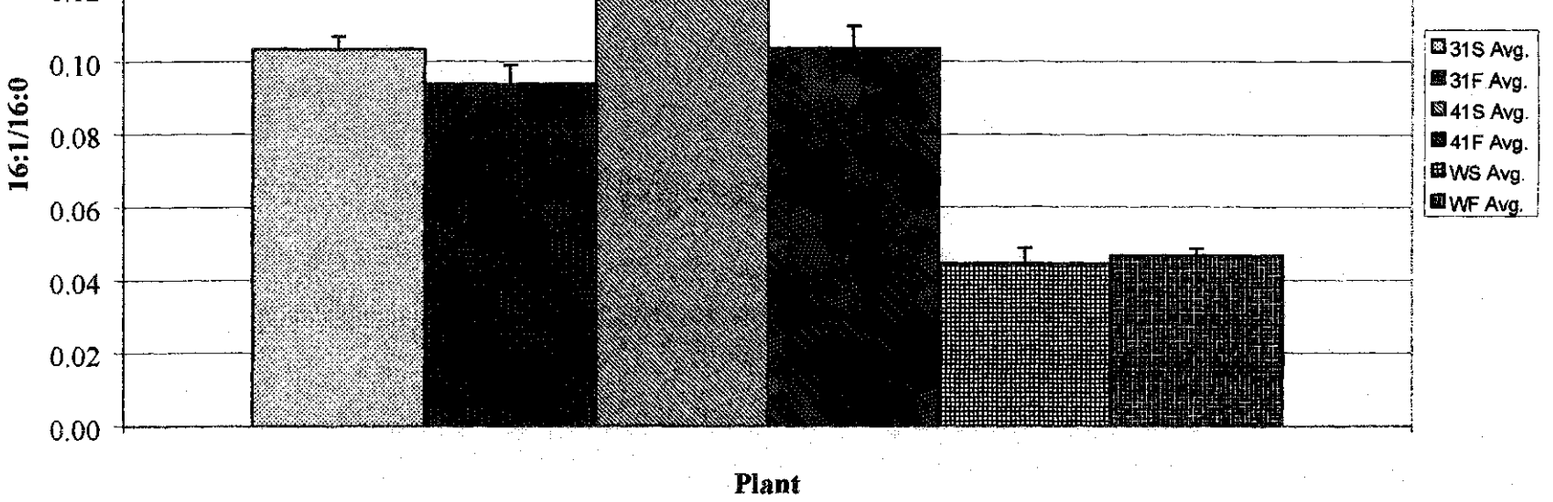

Figure 32. Comparison of $^{\Delta 9}$16:1/16:0 ratio of seed oil from *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase and control *B. napus* - summer and fall 2001 plants. The samples were compared using ANOVA testing and included C_2-31A grown in the summer (n=7) to C_2-31A grown in the fall (n=12), also C_2-41 grown in the summer (n=10) to C_2-41 grown in the fall (n=15), and lastly, Westar grown in the summer (n=12), to Westar grown in the fall (n=15), all significant differences are indicated with a *.

of $^{\Delta 9,12}$18:2 and significant reductions in the amount of $^{\Delta 9,12,15}$18:3 present in all plant lines grown in the summer.

4.2.9. Analysis of the Acyl-CoA pool of Developing *B.* napus Seed Transformed with cDNA Encoding Cat's Claw 16:0-ACP Desaturase

The FA composition of the acyl-CoA pool in the transgenic plant line transformed with cDNA encoding cat's claw 16:0-ACP was determined to assess whether endogenous AT selectivity limited the incorporation of certain FAs into TAG. Cat's claw seeds are known to contained nearly 80% $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1 in the seed oil and therefore, probably have the proper enzyme requirements for these FAs to be incorporated to TAG (Cahoon et al. 1998). The current acyl-CoA pool study showed that at 4 weeks PA there was much less SFA present in the acyl-CoA pool than that of the untransformed Westar (Figure 33). This increase in SFA of the untransformed Westar was a result of the very high amount of 18:0 present in the acyl-CoA pool, and may be explained based on the developmental profile of $^{\Delta 9}$18:0-ACP desaturase mRNA. Slocombe et al. (1992, 1994) reported that that mRNA was just detectable at 25 DPA, peaking at 45 DPA and declining thereafter. No statistical analysis were performed on our data because there was very limited sample for the untransformed Westar control, where the seeds were pooled and n=1. Also, the GLC detection method used here was not as sensitive as a fluorescence based method (Larson & Graham 2001). Here, the seeds from each transformed plant line were also pooled, but in this case n=3 for each.

Other trends in the acyl-CoA pool included an increase in all other USFAs detected in the transformed lines compared to the untransformed Westar control. Two of these included the $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1 which were present only in the transformed plant lines, and not in the Westar control. Also, in the transformed plant lines, there was a much reduced proportion of 16:0 present. There were large differences in the $^{\Delta 9}$16:1/16:0

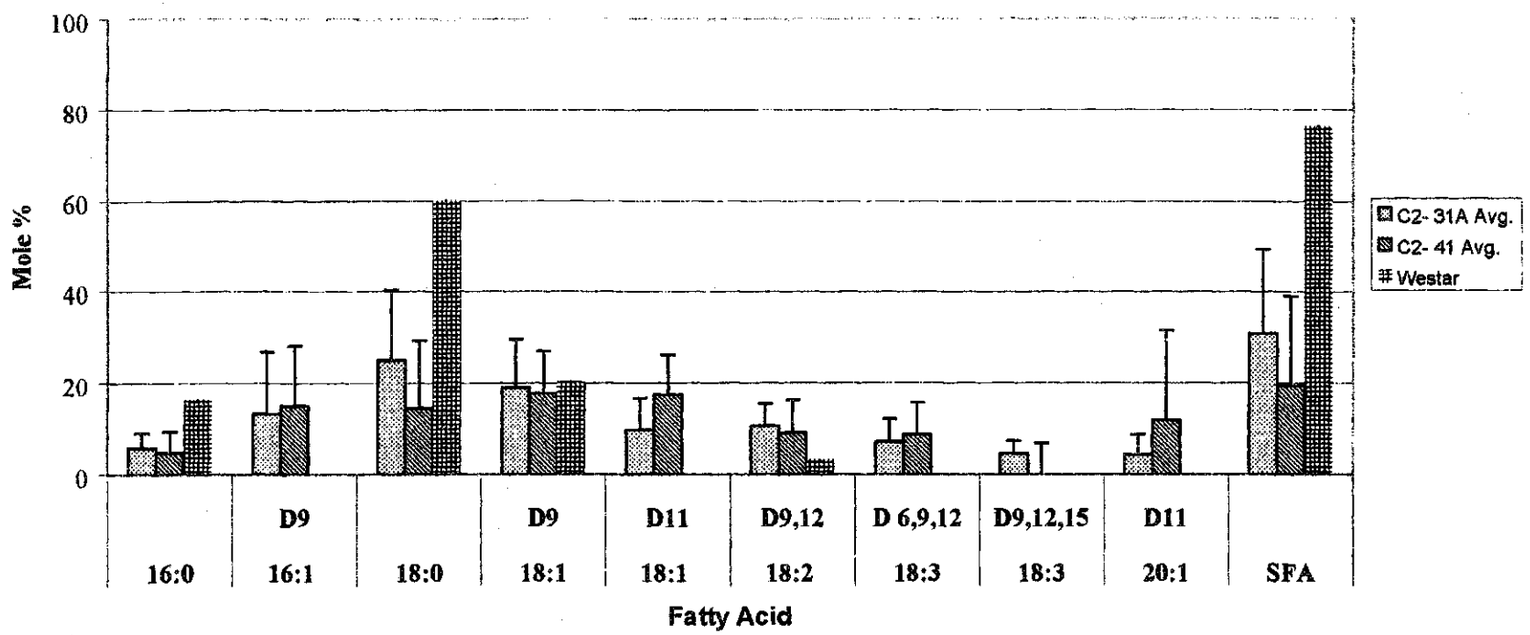

Figure 33. The FA composition (mole%) of the acyl-CoA pool in four week PA transformed *B. napus* containing a cDNA encoding cat's claw 16:0-ACP desaturase. The acyl-CoA pool was extracted from pooled four week PA developing seeds of *B. napus*, methylated, and run on a GLC. The lines include transformed lines C_2-31A (n=3), C_2-41 (n=3), and the untransformed Westar (n=1).

ratios in both C_2-31A and C_2-41 present in the acyl-CoA pool study 4 weeks post anthesis and the FA present in the mature seeds (Figure 34). The $^{\Delta 9}$16:1/16:0 ratios were almost 25 and 30 times higher in the acyl-CoA pool 4 weeks post anthesis than in the total acyl lipids of mature transformed lines C_2-31A and C_2-41, respectively. There was no 16:0 present in the Westar control of the acyl-CoA pool. Therefore, this ratio could not be calculated. The high ratio of the $^{\Delta 9}$16:1/16:0 in the acyl-CoA pool compared to the mature FA in the total lipids suggested that AT selectivity problem limited the incorporation of $^{\Delta 9}$16:1 into TAG. This is also seen when comparing the major acyl-CoA pool FAs to that of the mature FAs in the total lipids (Figure 35). Again this indicates that the plant limits the amount of both $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1, as there were much higher amounts of these FAs present in the acyl-CoA fraction than there is in the FAs of the mature seeds. The FAs 16:0, 18:0 and $^{\Delta 9}$18:1 were all present in the WT *B. napus* and there were almost no differences in the amounts of these FAs in the total acyl lipid of seed from the transformed and non-transformed plants, while there were greater differences in the amount of these FAs in the acyl-CoA pool.

A very recent study supports our observations and prediction that AT selectivity may limit achieving the desired FA composition in transgenic lines. Larson et al. (2002) transformed *B. napus* in an attempt to change the FA profile with many transgenes. A medium chain TE and KAS transgenes from *Cuphea hookeriana* were transformed into *B. napus* in an attempt to produce seed oil with medium and high 8:0 and 10:0 content, as *C. hookeriana* contains up to 50% 8:0 and 25% 10:0 in it's oil. The groups also made transformation to over produced 12:0 by transforming *B. napus* with California Bay TE, another FA not found in the FA profile of *B. napus*. Lastly, 18:0, an endogenous FA to

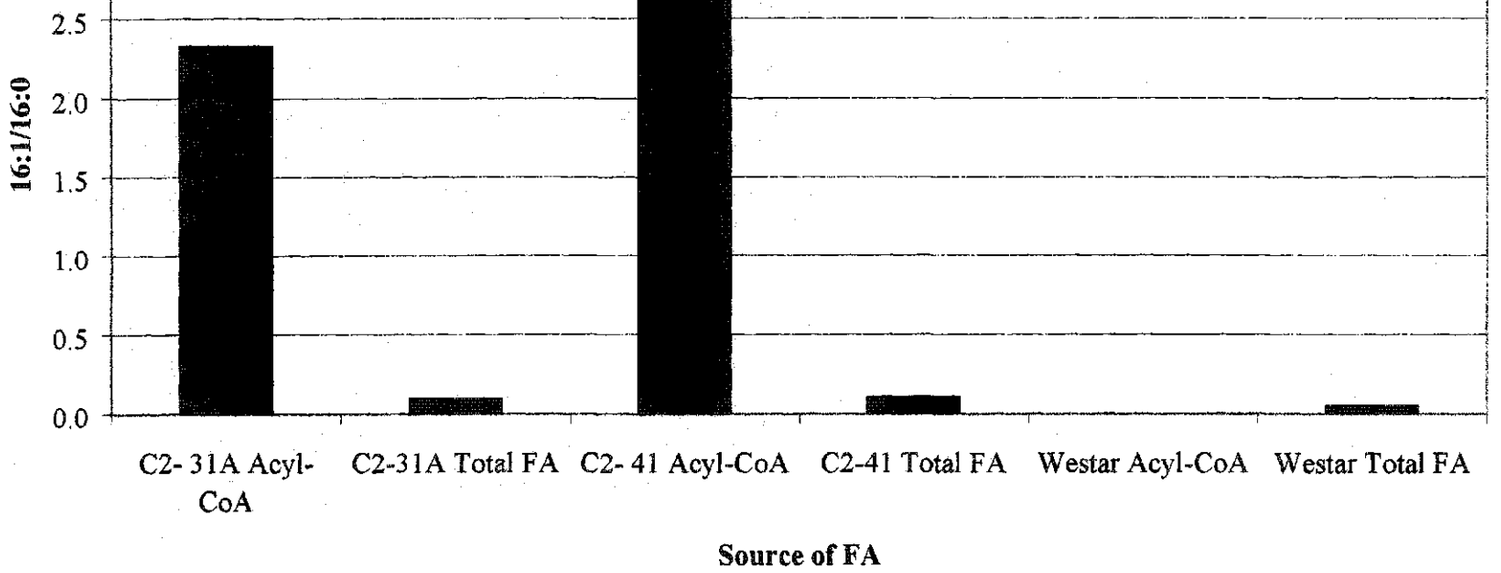

Figure 34. Comparison of $^{\Delta 9}$16:1/16:0 ratio in the acyl-CoA pool of four week PA and mature total lipids from *B. napus* containing a cDNA encoding cat's claw 16:0-ACP desaturase.

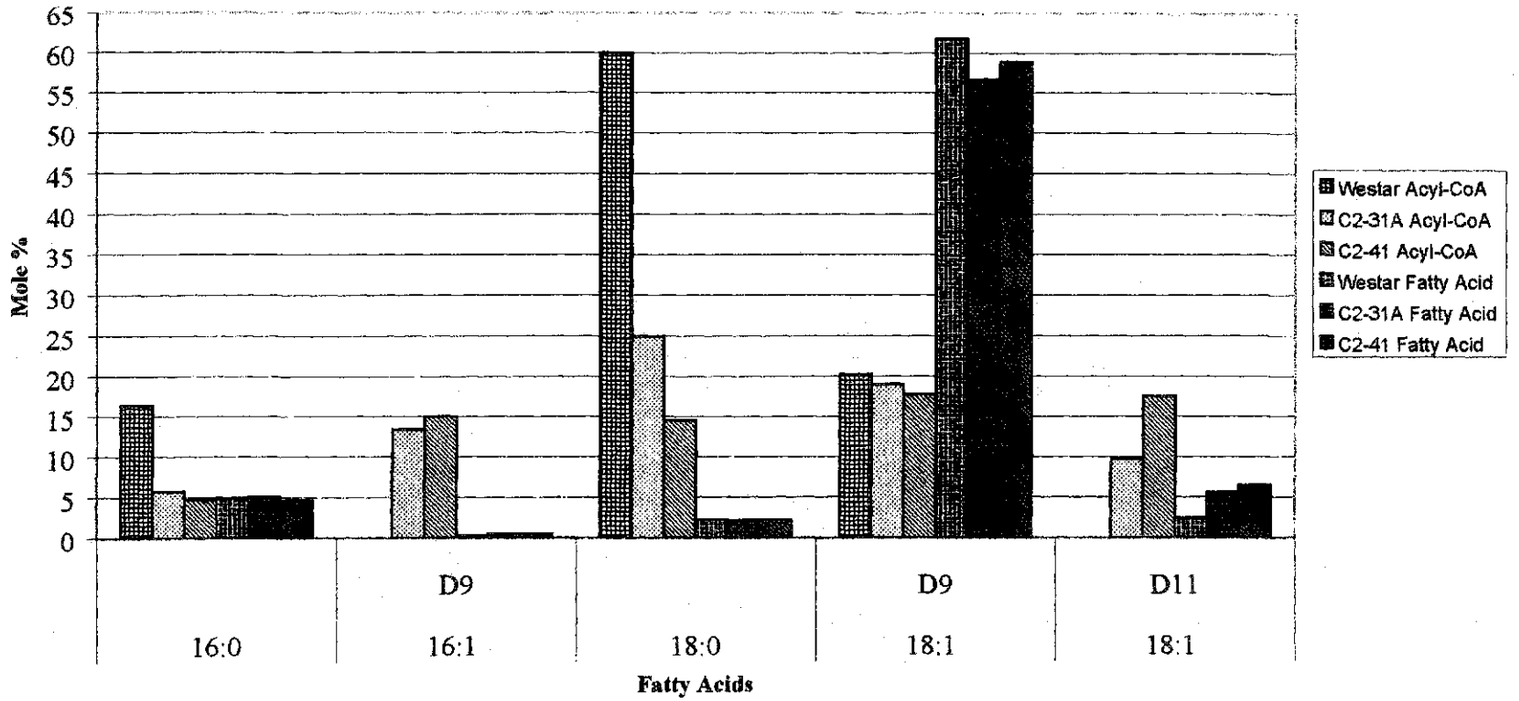

Figure 35. Comparison of the major FAs (mole%) in the acyl-CoA pool of developing and total lipids of mature *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. Acyl-CoAs were extracted from pooled samples of four week-PA developing *B. napus*. The acyl-CoA samples included Westar (n=1), C_2-31A (n=3), C_2-41(n=3), whereas the mature seeds total lipids included Westar (n=15), C_2-31A (n=12), C_2-41 (n=15).

B. napus, was over expressed and a null control line was produced. The results of the transformations showed that the activity of marker enzymes for β-oxidation or the glyoxylate cycle did not increase above background, and therefore, degradation of FAs were eliminated as a reason for the limitation of unusual FAs in TAG. The group did find that the acyl-CoA and lipid FA profiles were very different. In medium and high expressing lines, the 10:0-CoA and FA profiles were markedly different by 40 DPA compared to the null and WT lines. There was 47 mole% of 10:0-CoA and only 22 mole% of 10:0 FA in the high 8:0/10:0 line at 40 DPA. Altered profiles were also observed for 12:0 and overproducing 18:0 lines. Here, there was 61 mole% of 12:0-CoA and 55 mole% FA by 40 DPA, and 8.2 mole% 18:0-CoA and 20 mole% 18:0 FA. These numbers revealed that the acyl-CoA content does not necessarily correlate to the FA%. Also, due to highly sensitive techniques, the group measured the total acyl-CoA and lipid FA pool sizes, on a fresh weight basis. Here they found that in both the 8:0/10:0 and the 12:0 lines, the total acyl-CoA pool was significantly higher than the pool size of the WT seeds, whereas in the overproducing 18:0 line there was a significantly lower pool size than that of the WT. In contrast, while the acyl-CoA pools of the 8:0/10:0 and 12:0 producing lines were elevated, the total lipid FA pool size was significantly decreased from the WT line by 40 DPA, while in the overproducing 18:0 line there was no difference in the FA content between the transgenic and the WT lines. These results suggested that there were higher amounts of medium chain CoAs in the cytosol, or the medium chain FAs compromised the overall lipid accumulation process. This was explained based on a lack of AT activity towards the unusual acyl-CoA produced.

Clearly, just because a plant has the ability to produce a FA, that FA may not always appear within the oil and AT limitations may be the underlying cause.

5.0 Summary and Future Directions

The main purpose of this thesis was to determine if the SFA content of *B. napus* seed oil could be reduced by transforming the oilseed crop with a cDNA encoding cat's claw 16:0-ACP desaturase. Some experiments were also conducted with *A. thaliana*, the model Crucifer. Lipid analyses were conducted with seed and leaf tissue from *A. thaliana* transformed with the intention of ubiquitously expressing the cat's claw desaturase. Transformations with *B. napus* were aimed at seed-specific expression of the enzyme. Mature seed from *B. napus* transformants were analyzed for cDNA inserts, cat's claw desaturase transcript and seed lipid FA composition. As well, developing *B. napus* seeds were analyzed for the FA composition of the acyl-CoA pool. In *A. thaliana*, transformed plants were identified that had seed lipid $^{\Delta 9}$16:1/16:0 ratios that were significantly higher than the untransformed control. Only one transformed plant line, 16-1, had an increase in $^{\Delta 9}$16:1/16:0 in the leaf total lipids. In all fractions tested, the elongated product of $^{\Delta 9}$16:1, $^{\Delta 11}$18:1, had increased significantly in the transformants. These increases in $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1 suggested that the cat's claw 16:0-ACP desaturase was functionally expressed in *A. thaliana.* For *B. napus*, the results of all the gene identification tests showed that the transformed plants contained at least 2 copies of the cDNA encoding cat's claw 16:0-ACP desaturase. The level of transcript, encoding the cat's claw desaturase, was greater in *B. napus* transformants C_2-41-6 and C_2-41-11, compared to C_2-31A-1 and C_2-31A-10. In both summer and fall, two greenhouse trials on two promising lines indicated that there were increased amounts of $^{\Delta 9}$16:1 and the elongation product, $^{\Delta 11}$18:1, produced in the seed oil. The increase in $^{\Delta 9}$16:1 and decreases in 16:0 in the C_2-31A in the summer and C_2-41 in the fall caused $^{\Delta 9}$16:1/16:0

ratios to be increased significantly for both transformed lines compared to the control in both summer and fall. In both cases, the C_2-41 line had a larger $^{\Delta 9}$16:1/16:0 ratio than the C_2-31A line suggesting that the cat's claw desaturase was more active within the C_2-41 transformant. A number of other changes in FA composition of seed oil were observed. The seed oil from *B. napus* grown in the first greenhouse trial, during the summer, showed a significantly increased SFA content for both transformants compared to the control, and involved increases in almost all SFAs present. In contrast, seed oil from *B. napus* grown in the greenhouse in the fall had no significant changes in the amount of SFA present in the seed oil of the transformants compared to the untransformed control. The greenhouse temperatures in the summer trial were considerably greater than for fall trial suggesting that the transformants exhibiting an increase in SFA in response to higher temperatures than control plants. The investigation of the acyl-CoA pool of developing seeds of *B. napus* transformants revealed increases in all USFAs when compared to the untransformed control. Two of these FAs included $^{\Delta 9}$16:1 and $^{\Delta 11}$18:1, which probably represented the product of the catalytic action of 16:0-ACP desaturase and the elongation product, respectively. Also, the transformants displayed a reduced mole% of 16:0. The $^{\Delta 9}$16:1/16:0 ratios of the acyl-CoA pools of developing seeds (4 weeks post anthesis) of transformants C_2-31A and C_2-41 were about 25 and 30 times higher, respectively, than for the mature seeds of the two lines. This observation indicated that AT acyl-CoA selectivity might have placed limits on the incorporation of 16:1 into seed TAG because there appears to be more 16:1 avaliable as CoA then there is 16:1 present in TAG.

Further insight into the relationship between the FA composition of the acyl-CoA pool of developing seeds and the FA composition of TAG from mature seed of *B. napus*

transformants will come from analyses of the acyl-CoA pool at different stages of seed development. Substantial decreases in SFA content of *B. napus* transformed with the cDNA encoding cat's claw 16:0-ACP desaturase may only be realized if the transformants developed in this study are crossed with transformants containing AT activity with an enhanced selectivity for $^{\Delta 9}$16:1. Studies on the positional distribution of FAs on TAG and selectivity properties of the ATs involved in synthesizing TAG, with respect to utilizing $^{\Delta 9}$16:1-CoA and acceptor substrates containing $^{\Delta 9}$16:1 moieties, should reveal the enzymes that need to be modified. It would also be interesting to further investigate $^{\Delta 9}$16:1 production in the leaf lipids of *A. thaliana* transformed to ubiquitously express the cat's claw desaturase. A low temperature challenge of these transformants may increase the production of $^{\Delta 9}$16:1 in the leaves and lead to the additional benefit of increased membrane fluidity in the leaves at lower temperatures. The investigation should be coupled with the analysis of the PG fraction. In the long term, it may be possible to develop a genetically engineered line of canola with enhanced chilling tolerance and decreased content of USFAs in the seed oil.

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6.0 APPENDIX I

Table 1. FA composition (mole%) of seed total acyl lipid from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The total acyl lipids were extracted using HIP extraction, followed by methylation with methanolic-HCl and FAMES were run on a GLC as described in MATERIALS AND METHODS. ANOVA tests were performed comparing the transformed lines, 16-1, 26-1, and 27-1 to the untransformed WS control. All significant P-values ($p<0.07$) are in **bold**. For all transformed lines and control $n=5$.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ6,9,12	18:3Δ 9,12,15	20:0	20:1 Δ11	20:2 Δ11,14	20:3Δ 11,14,17	22:0	22:1 Δ13	24:0	SFA
16-1 TL Avg	0.03	6.91	1.37	9.00	12.40	7.48	24.78	0.48	14.72	4.13	15.08	1.47	0.31	0.36	1.37	0.12	20.56
16-1 TL S.E.	0.03	0.02	0.01	0.15	0.05	0.07	0.10	0.02	0.11	0.06	0.07	0.01	0.00	0.01	0.01	0.00	0.21
P >F 16-1 TL	0.35	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.35	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
26-1 TL Avg	0.03	8.55	1.33	8.99	12.87	6.17	25.26	0.57	13.90	3.34	15.32	1.40	0.37	0.32	1.37	0.21	21.44
26-1 TL S.E.	0.03	1.39	0.17	0.88	1.07	0.16	0.68	0.07	0.30	0.08	0.41	0.04	0.08	0.01	0.01	0.10	2.31
P >F 26-1 TL	0.35	0.47	**0.00**	**0.06**	**0.01**	**0.00**	**0.01**	0.35	0.25	**0.00**	**0.00**	**0.01**	0.33	**0.00**	**0.00**	0.30	0.12
27-1 TL Avg	0.03	7.16	1.22	9.19	12.97	5.36	25.63	0.46	14.91	4.14	15.30	1.49	0.31	0.35	1.36	0.12	20.99
27-1 TL S.E.	0.03	0.10	0.02	0.24	0.19	1.34	0.31	0.04	0.22	0.11	0.20	0.02	0.01	0.01	0.02	0.01	0.41
P >F 27-1 TL	0.35	0.01	**0.00**	**0.00**	**0.00**	**0.02**	**0.00**	0.29	0.04	**0.00**	**0.00**	0.05	**0.00**	**0.00**	**0.00**	**0.01**	**0.00**
WS TL Avg	0.00	7.49	0.26	7.07	16.84	1.52	27.56	0.51	14.30	2.55	18.21	1.55	0.28	0.25	1.50	0.10	17.47
WS TL S.E.	0.00	0.03	0.00	0.07	0.07	0.01	0.04	0.02	0.10	0.02	0.05	0.01	0.00	0.00	0.01	0.00	0.08

Table 2. FA composition (mole%) of seed TAG from *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The TAG lipids were extracted from the seed total lipid via the HIP extraction and separation of TAG using TLC in the solvent system of hexane:diethyl ether:glacial acetic acid (80:20:1) (v/v/v) against the a 19:1TAG standard. The TAG portion was methlyated using methanolic-HCL and FAMES were run on a GLC as described in MATERIALS AND METHODS. The transformed lines, 16-1, 26-1, and 27-1, were compared to the untransformed control WS. ANOVA tests were performed and all significant P-values ($p < 0.07$) are in **bold.** For all transformed lines and controls $n=5$.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ6,9,12	18:3Δ 9,12,15	20:0	20:1 Δ11	20:2 Δ11,14	20:3Δ 11,14,17	22:0	22:1 Δ13	24:0	SFA
16-1 TAG Avg	0.00	7.10	0.99	21.03	14.57	6.75	18.48	1.83	5.87	5.06	15.34	1.08	0.08	0.41	1.30	0.12	33.72
16-1 TAG S.E.	0.00	0.43	0.25	8.62	1.11	1.04	3.39	1.05	1.92	0.32	1.52	0.28	0.05	0.11	0.33	0.03	7.97
p >F 16-1	0.35	0.29	**0.01**	0.98	**0.04**	**0.00**	0.99	0.75	0.49	**0.01**	0.14	0.91	0.41	0.64	0.22	**0.04**	0.90
26-1 TAG Avg	0.00	8.33	1.12	11.14	15.10	6.36	23.49	0.83	9.74	4.16	16.20	1.33	0.17	0.45	1.47	0.11	24.20
26-1 TAG S.E.	0.00	0.06	0.03	1.19	0.63	0.10	1.09	0.17	1.40	0.21	0.26	0.06	0.04	0.02	0.04	0.03	1.43
p >F 26-1	-	0.43	**0.00**	0.26	**0.04**	**0.00**	0.25	0.25	**0.02**	**0.07**	0.13	0.37	**0.03**	0.30	**0.06**	**0.04**	0.30
27-1 TAG Avg	0.00	7.08	0.82	28.21	15.91	5.11	13.80	3.09	2.70	5.41	14.98	0.83	0.00	0.51	1.46	0.10	41.31
27-1 TAG S.E.	0.00	0.53	0.25	10.67	2.57	1.29	3.64	1.74	0.90	0.58	2.59	0.22	0.00	0.14	0.37	0.06	9.64
p >F 27-1	-	0.31	**0.03**	0.58	0.28	**0.02**	0.41	0.74	0.43	**0.02**	0.23	0.61	0.35	0.33	0.46	0.30	0.48
WS TAG Avg.	0.00	7.89	0.15	20.69	19.47	1.32	18.42	2.36	4.14	3.46	18.92	1.03	0.03	0.34	1.76	0.02	32.39
WS TAG S.E.	0.00	0.54	0.06	7.78	1.63	0.33	3.94	1.24	1.47	0.27	1.59	0.31	0.03	0.10	0.12	0.02	7.18

Table 3. Comparison of the FA composition (mole%) in the seed total acyl lipids and TAG in *A. thaliana* transformed with a cDNA encoding a cat's claw 16:0-ACP desaturase. ANOVA testing was performed and all significant P-values ($p < 0.07$) are in **bold.** For all samples $n=5$.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ6,9,12	18:3Δ 9,12,15	20:0	20:1 Δ11	20:2 Δ11,14	20:3Δ 11,14,17	22:0	22:1 Δ13	24:0	SFA	16:1/ 16:0
16-1 TL Avg.	0.03	6.91	1.37	9.00	12.40	7.48	24.78	14.72	0.48	4.13	15.08	1.47	0.31	0.36	1.37	0.12	20.56	0.20
16-1 TL S.E.	0.03	0.02	0.01	0.15	0.05	0.07	0.10	0.11	0.02	0.06	0.07	0.01	0.00	0.01	0.01	0.00	0.21	0.00
16-1 TAG Avg.	0.00	7.10	0.99	21.03	14.57	6.75	18.48	5.87	1.83	5.06	15.34	1.08	0.08	0.41	1.30	0.12	33.72	0.13
16-1 TAG S.E.	0.00	0.43	0.25	8.62	1.11	1.04	3.39	1.92	1.05	0.32	1.52	0.28	0.05	0.11	0.33	0.03	7.97	0.03
P >F	0.35	0.67	0.17	0.20	0.09	0.51	0.10	**0.00**	0.23	**0.02**	0.87	0.20	0.00	0.67	0.84	0.97	0.14	0.08
26-1 TL Avg.	0.03	8.55	1.33	8.99	12.87	6.17	25.26	13.90	0.57	3.34	15.32	1.40	0.37	0.32	1.37	0.21	21.44	0.16
26-1 TL S.E.	0.03	1.39	0.17	0.88	1.07	0.16	0.68	0.30	0.07	0.08	0.41	0.04	0.08	0.01	0.01	0.10	2.31	0.01
26-1 TAG Avg.	0.00	8.33	1.12	11.14	15.10	6.36	23.49	9.74	0.83	4.16	16.20	1.33	0.17	0.45	1.47	0.11	24.20	0.13
26-1 TAG S.E.	0.00	0.06	0.03	1.19	0.63	0.10	1.09	1.40	0.17	0.21	0.26	0.06	0.04	0.02	0.04	0.03	1.43	0.00
P >F	0.35	0.88	0.26	0.18	0.11	0.33	0.21	**0.02**	0.21	**0.01**	0.11	0.37	**0.06**	**0.00**	**0.05**	0.37	0.34	**0.01**
27-1 TL Avg.	0.03	7.16	1.22	9.19	12.97	5.36	25.63	14.91	0.46	4.14	15.30	1.49	0.31	0.35	1.36	0.12	20.99	0.17
27-1 TL S.E.	0.03	0.10	0.02	0.24	0.19	1.34	0.31	0.22	0.04	0.11	0.20	0.02	0.01	0.01	0.02	0.01	0.41	0.00
27-1 TAG Avg.	0.00	7.08	0.82	28.21	15.91	5.11	13.80	2.70	3.09	5.41	14.98	0.83	0.00	0.51	1.46	0.10	41.31	0.11
27-1 TAG S.E.	0.00	0.53	0.25	10.67	2.57	1.29	3.64	0.90	1.74	0.58	2.59	0.22	0.00	0.14	0.37	0.06	9.64	0.03
P >F	0.35	0.89	0.15	0.11	0.29	0.90	0.01	**0.00**	0.17	**0.06**	0.90	**0.02**	**0.00**	0.28	0.81	0.70	**0.07**	**0.07**
WS TL Avg.	0.00	7.49	0.26	7.07	16.84	1.52	27.56	14.30	0.51	2.55	18.21	1.55	0.28	0.25	1.50	0.10	17.47	0.03
WS TL S.E.	0.00	0.03	0.00	0.07	0.07	0.01	0.04	0.10	0.02	0.02	0.05	0.01	0.00	0.00	0.01	0.00	0.08	0.00
WS TAG Avg.	0.00	7.89	0.15	20.69	19.47	1.32	18.42	4.14	2.36	3.46	18.92	1.03	0.03	0.34	1.76	0.02	32.39	0.02
WS TAG S.E.	0.00	0.54	0.06	7.78	1.63	0.33	3.94	1.47	1.24	0.27	1.59	0.31	0.03	0.10	0.12	0.02	7.18	0.01
P >F	-	0.48	0.15	0.12	0.15	0.57	**0.05**	**0.00**	0.17	**0.01**	0.67	0.13	**0.00**	0.41	**0.07**	**0.01**	**0.07**	**0.05**

Table 4. The 16:1/16:0 ratio for *A. thaliana* transformed with a cDNA encoding a cat's claw 16:0-ACP desaturase. The three transformed lines include 16-1, 26-1, and 27-1, and were compared to the untransformed control, WS, using ANOVA analysis. All significant P-values ($p < 0.07$) are shown in **bold.** For all transformed lines and the control $n=5$.

	Leaf Total Lipids	Seed Total Lipids	Seed TAG
16-1 Avg	0.03	0.20	0.13
16-1 S.E.	0.00	0.00	0.03
P >F	**0.04**	**0.00**	**0.01**
26-1 Avg.	0.01	0.16	0.13
26-1 S.E.	0.01	0.01	0.00
P >F	0.92	**0.00**	**0.00**
27-1 Avg.	0.02	0.17	0.11
27-1 S.E.	0.01	0.00	0.03
P >F	0.44	**0.00**	**0.02**
WS Avg.	0.01	0.03	0.02
WS S.E.	0.01	0.00	0.01

Table 5. FA composition (mole%) of total acyl lipids from leaves of *A. thaliana* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase. The total acyl lipids were extracted using HIP extraction, followed by methylation with methanolic-HCl and FAMES were run on a GLC as described in MATERIALS AND METHODS. ANOVA tests were performed comparing the transformed lines, 16-1, 26-1, and 27-1 to the untransformed WS control. All significant P-values ($p < 0.07$) are in **bold.** For all transformed lines and control n=5.

	14:0	16:0	16:1 Δ9	16:3** Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ6,9,12	18:3Δ 9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA
16-1	0.02	20.81	0.59	8.38	11.56	4.73	3.55	13.61	1.36	33.05	0.83	0.00	0.71	0.81	34.74
16-1 S.E.	0.00	0.55	0.04	1.72	0.54	0.37	0.30	0.26	0.11	1.16	0.03	0.00	0.03	0.02	0.95
p >F	0.33	0.24	**0.05**	0.94	**0.04**	**0.03**	**0.00**	**0.00**	0.39	0.51	**0.00**	-	**0.04**	**0.07**	0.35
26-1 Avg.	0.02	21.04	0.29	10.59	12.24	6.58	2.80	12.97	2.27	29.00	0.75	0.00	0.65	0.79	35.50
26-1 S.E.	0.00	0.68	0.12	0.82	1.04	1.37	0.20	0.26	0.77	1.28	0.02	0.00	0.02	0.03	0.92
p >F	0.27	0.48	0.96	**0.07**	0.08	0.60	**0.00**	**0.00**	0.22	**0.06**	**0.01**	-	0.17	0.19	0.11
27-1 Avg.	0.02	20.22	0.41	8.83	11.40	4.72	3.07	14.13	1.90	33.18	0.75	0.05	0.60	0.72	33.71
27-1 S.E.	0.00	0.51	0.12	0.54	0.25	0.29	0.29	0.36	0.51	0.55	0.02	0.05	0.02	0.01	0.40
p >F	0.53	**0.05**	0.51	0.70	0.01	**0.03**	**0.00**	**0.03**	0.23	0.25	**0.01**	0.35	0.64	0.50	0.99
WS Avg.	0.02	21.59	0.29	8.52	10.15	7.50	0.95	15.64	1.25	32.14	0.64	0.00	0.58	0.74	33.71
WS S.E.	0.00	0.30	0.12	0.56	0.22	0.97	0.05	0.46	0.05	0.65	0.02	0.00	0.05	0.02	0.38

Table 9. FA composition (mole%) of seed total acyl lipid from *B. napus* containing a cDNA cat's claw 16:0-ACP desaturase - summer 2001 plants. The two transformed lines C_2-31A (n=7) and C_2-41 (n=10) were compared to Westar (n=12), where the total acyl lipids were extracted using the PE extraction, followed by methylation with NaMeth and FAMES were run on GLC as described in MATERIALS AND METHODS. ANOVA testing was performed with significant P-values ($p< 0.07$) in **bold**.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A Avg	0.07	4.45	0.46	3.23	62.94	5.99	15.11	4.98	0.97	1.11	0.43	0.27	9.42	0.10
C_2-31A S.E.	0.00	0.07	0.01	0.18	0.75	0.26	0.38	0.12	0.04	0.02	0.01	0.01	0.25	0.00
p>F	0.53	**0.06**	**0.00**	**0.00**	**0.00**	**0.00**	**0.01**	**0.02**	**0.00**	0.43	**0.00**	**0.00**	**0.00**	**0.00**
C_2-41A Avg.	0.09	4.55	0.62	4.14	56.12	8.52	17.60	5.67	1.05	0.98	0.41	0.25	10.49	0.14
C_2-41 S.E.	0.00	0.21	0.05	0.24	1.81	0.70	0.71	0.30	0.05	0.03	0.02	0.01	0.51	0.01
p>F	0.62	0.23	**0.00**	**0.00**	**0.00**	**0.00**	**0.05**	**0.00**	**0.00**	**0.00**	**0.01**	**0.02**	**0.00**	**0.00**
Westar Avg.	0.12	4.31	0.19	2.65	67.32	2.25	16.20	4.62	0.76	1.10	0.29	0.19	8.32	0.04
Westar S.E.	0.06	0.03	0.02	0.08	0.36	0.21	0.21	0.07	0.02	0.01	0.03	0.02	0.10	0.00

Table 10. FA composition (mole%) of total acyl lipids of seeds from *B. napus* containing cDNA encoding cat's claw 16:0-ACP desaturase- fall 2001 plants. The total acyl lipids from mature T_2 seeds were extracted via PE extraction, followed by methylated by NaM, and FAMES were run on a GLC as described by MATERIALS AND METHODS. The transformed lines, C_2-31A (n=12) and C_2-41 (n=15), were compared to the untransformed and control *B. napus* cv L. Westar (n=15). Statistical analysis was performed by ANOVA testing and all significant P-values ($p < 0.07$) are in **bold**.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A Avg.	0.03	5.13	0.48	2.18	56.70	5.63	18.96	7.98	0.85	1.24	0.55	0.26	9.01	0.09
C_2-31A S.E.	0.01	0.10	0.02	0.10	1.09	0.24	0.64	0.24	0.03	0.02	0.02	0.01	0.22	0.00
P>F	0.17	0.37	**0.00**	0.66	**0.00**	**0.00**	0.57	**0.00**	0.83	0.39	0.30	0.48	0.88	**0.00**
C_2-41 Avg.	0.06	4.71	0.49	2.14	58.84	6.55	17.96	6.64	0.75	1.15	0.47	0.23	8.37	0.10
C_2-41 S.E.	0.04	0.10	0.02	0.10	0.89	0.30	0.51	0.15	0.05	0.02	0.02	0.01	0.26	0.00
P>F	0.89	**0.04**	**0.00**	0.46	**0.02**	**0.00**	0.48	0.56	0.12	**0.00**	**0.03**	**0.01**	0.11	**0.00**
Westar Avg.	0.05	5.01	5.01	2.24	61.86	2.43	18.47	6.76	0.85	1.26	0.53	0.28	8.99	0.05
Westar S.E.	0.01	0.09	0.09	0.09	0.98	0.21	0.57	0.21	0.03	0.02	0.01	0.01	0.19	0.00

Table 11. Comparison of FA composition (mole%) of seed total lipids from *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase- summer and fall 2001 plants. FAs were extracted using the PE extraction, followed by methylation with NaMeth and FAMES were run on a GLC as described in the MATERIALS AND METHODS. ANOVA testing was used and significant P-values ($p < 0.07$) are in **bold**.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A S Avg.	0.07	4.45	0.46	3.23	62.94	5.99	15.11	4.98	0.97	1.11	0.43	0.27	9.42	0.10
C_2-31A S S.E.	0.00	0.07	0.01	0.18	0.75	0.26	0.38	0.12	0.04	0.02	0.01	0.01	0.25	0.00
C_2-31A F Avg.	0.03	5.13	0.48	2.19	56.70	5.63	18.96	7.98	0.85	1.24	0.55	0.26	9.01	0.09
C_2-31A F S.E.	0.01	0.07	0.03	0.09	1.16	0.32	0.70	0.28	0.03	0.02	0.01	0.01	0.14	0.01
P>F	**0.03**	**0.00**	0.56	**0.00**	**0.00**	0.45	**0.00**	**0.00**	**0.01**	**0.00**	**0.00**	0.61	0.13	0.22
C_2-41 S Avg.	0.09	4.55	0.62	4.14	56.12	8.52	17.60	5.67	1.05	0.98	0.41	0.25	10.49	0.14
C_2-41 S S.E.	0.00	0.21	0.05	0.24	1.81	0.70	0.71	0.30	0.05	0.03	0.02	0.01	0.51	0.01
C_2-41 F Avg.	0.06	4.71	0.49	2.14	58.84	6.55	17.96	6.64	0.75	1.15	0.47	0.23	8.37	0.10
C_2-41 F S.E.	0.06	0.09	0.03	0.11	0.84	0.40	0.50	0.10	0.06	0.02	0.02	0.01	0.28	0.01
P>F	0.73	0.45	**0.02**	**0.00**	0.14	**0.02**	0.67	**0.00**	**0.00**	**0.00**	**0.03**	0.43	**0.00**	**0.00**
WS Avg.	0.12	4.31	0.19	2.65	67.32	2.25	16.20	4.62	0.76	1.10	0.29	0.19	8.32	0.04
WS S.E.	0.06	0.03	0.02	0.08	0.36	0.21	0.21	0.07	0.02	0.01	0.03	0.02	0.10	0.00
WF Avg.	0.05	5.01	0.24	2.24	61.86	2.43	18.47	6.76	0.85	1.26	0.53	0.28	8.97	0.05
WF S.E.	0.01	0.11	0.01	0.09	0.94	0.13	0.52	0.18	0.03	0.02	0.02	0.01	0.24	0.00
P>F	0.21	**0.00**	**0.07**	**0.00**	**0.00**	0.43	**0.00**	**0.00**	**0.01**	**0.00**	**0.00**	**0.00**	**0.03**	0.62

Table 12. Differences in FA composition (mole%) in *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase using the PE extraction or the HIP extraction followed by methylation with NaMeth-summer 2001 plants. Once FAMES were obtained they were run on a GLC as described in MATERIALS AND METHODS. Samples included C_2-31A (n=7) and C_2-41 (n=10) and Westar (n=12). ANOVA testing was performed with all significant P-values ($p < 0.07$) shown in **bold**. Abbreviations in the table include P for the Pe, H for HIP and N for NaMeth.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A PN Avg.	0.07	4.45	0.46	3.23	62.94	5.99	15.11	4.98	0.97	1.11	0.43	0.27	9.42	0.10
C_2-31A PN S.E.	0.00	0.07	0.01	0.18	0.75	0.26	0.38	0.12	0.04	0.02	0.01	0.01	0.25	0.00
C_2-31A HN Avg.	0.06	4.37	0.46	3.06	62.72	6.13	15.27	5.24	0.93	1.10	0.41	0.25	9.09	0.11
C_2-31A HN S.E.	0.01	0.05	0.01	0.15	0.76	0.34	0.45	0.14	0.03	0.02	0.01	0.01	0.19	0.00
P>F	0.39	0.41	0.84	0.49	0.84	0.75	0.79	0.17	0.37	0.43	0.44	0.14	0.30	0.63
C_2-41 PN Avg.	0.09	4.55	0.62	4.14	56.12	8.52	17.60	5.67	1.05	0.98	0.41	0.25	10.49	0.14
C_2-41 PN S.E.	0.00	0.21	0.05	0.24	1.81	0.70	0.71	0.30	0.05	0.03	0.02	0.01	0.51	0.01
C_2-41 HN Avg.	0.16	4.38	0.62	4.07	56.11	8.88	17.80	5.32	1.04	0.98	0.41	0.22	10.29	0.14
C_2-41 HN S.E.	0.08	0.15	0.05	0.27	1.68	0.71	0.58	0.28	0.06	0.03	0.02	0.02	0.50	0.01
P>F	0.41	0.52	0.94	0.86	1.00	0.72	0.83	0.40	0.94	0.93	0.98	0.27	0.79	0.65
WS PN Avg.	0.12	4.31	0.19	2.65	67.32	2.25	16.20	4.62	0.76	1.10	0.29	0.19	8.32	0.04
WS PN S.E.	0.06	0.03	0.02	0.08	0.36	0.21	0.21	0.07	0.02	0.01	0.03	0.02	0.10	0.00
WS HN Avg.	0.07	4.19	0.20	2.51	67.29	2.42	16.33	4.67	0.73	1.10	0.32	0.19	8.01	0.05
WS HN S.E.	0.01	0.03	0.00	0.07	0.25	0.05	0.18	0.08	0.01	0.01	0.01	0.00	0.07	0.00
P>F	0.35	**0.01**	0.77	0.22	0.94	0.43	0.64	0.69	0.22	0.82	0.37	0.78	**0.02**	0.51

Table 13. Differences in FA composition (mole%) in *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase using the PE extraction or the HIP extraction followed by methylation with methanolic HCl-summer 2001 plants.

Once FAMES were obtained they were run on a GLC as described in MATERIALS AND METHODS. Samples included C_2-31A (n=7) and C_2-41 (n=10) and Westar (n=12). ANOVA testing was performed with all significant P-values ($p < 0.07$) shown in **bold**. Abbreviations in the table include P for the Pe, H for HIP and M for methanolic HCl.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A PM Avg.	0.00	4.29	0.38	7.03	59.68	6.40	14.63	4.77	1.01	1.08	0.46	0.28	13.06	0.09
C_2-31A PM S.E.	0.00	0.05	0.01	0.26	0.63	0.29	0.34	0.11	0.04	0.02	0.01	0.01	0.32	0.00
C_2-31A HM Avg.	0.09	4.31	0.44	4.34	61.50	6.30	15.13	5.16	0.94	1.09	0.44	0.26	10.38	0.10
C_2-31A HM S.E.	0.00	0.06	0.01	0.16	0.75	0.27	0.42	0.14	0.03	0.02	0.01	0.01	0.22	0.00
P>F	**0.00**	0.73	**0.02**	**0.00**	0.09	0.79	0.37	**0.04**	0.21	0.83	0.43	0.34	**0.00**	**0.04**
C_2-41 PM Avg.	0.00	4.75	0.58	8.06	52.36	9.05	17.08	5.50	1.03	0.93	0.42	0.25	14.51	0.12
C_2-41 PM S.E.	0.00	0.24	0.04	0.50	1.83	0.64	0.67	0.27	0.05	0.03	0.02	0.02	0.81	0.01
C_2-41 HM Avg.	0.00	4.38	0.57	8.83	52.49	9.03	17.08	5.00	1.04	0.92	0.41	0.23	14.90	0.13
C_2-41 HM S.E.	0.00	0.16	0.05	0.35	1.67	0.72	0.55	0.25	0.06	0.02	0.02	0.01	0.57	0.01
P>F	-	0.23	0.94	0.23	0.96	0.98	0.99	0.19	0.62	0.88	0.90	0.46	0.70	0.53
WS PM Avg.	0.00	4.21	0.16	6.54	63.84	2.63	15.67	4.56	0.78	1.05	0.34	0.21	12.09	0.04
WS PM S.E.	0.00	0.10	0.03	0.16	0.28	0.04	0.24	0.07	0.02	0.02	0.01	0.01	0.23	0.01
WS HM Avg.	0.02	4.17	0.11	6.48	64.10	2.47	15.84	4.47	0.75	1.05	0.34	0.20	11.95	0.03
WS HM S.E.	0.01	0.03	0.03	0.43	0.39	0.03	0.18	0.08	0.01	0.01	0.01	0.01	0.43	0.01
P>F	0.10	0.63	0.29	0.91	0.61	**0.00**	0.57	0.46	0.13	0.86	0.33	0.12	0.79	0.33

Table 14. Differences in FA composition (mole%) in *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase using the PE extraction followed by methylation with either NaMeth or methanolic HCl-summer 2001 plants. Once FAMES were obtained they were run on a GLC as described in MATERIALS AND METHODS. Samples included C_2-31A (n=7) and C_2-41 (n=10) and Westar (n=12). ANOVA testing was performed with all significant P-values ($p < 0.07$) shown in **bold.** Abbreviations in the table include P for the Pe, N for NaMeth or M for methanolic-HCl.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A PN Avg.	0.07	4.45	0.46	3.23	62.94	5.99	15.11	4.98	0.97	1.11	0.43	0.27	9.42	0.10
C_2-31A PN S.E.	0.00	0.07	0.01	0.18	0.75	0.26	0.38	0.12	0.04	0.02	0.01	0.01	0.25	0.00
C_2-31A PM Avg.	0.00	4.29	0.38	7.03	59.68	6.40	14.63	4.77	1.01	1.08	0.46	0.28	13.06	0.09
C_2-31A PM S.E.	0.00	0.05	0.01	0.26	0.63	0.29	0.34	0.11	0.04	0.02	0.01	0.01	0.32	0.00
P>F	**0.00**	0.09	**0.00**	**0.00**	**0.01**	0.30	0.37	0.22	0.48	0.18	0.20	0.91	**0.00**	**0.02**
C_2-41 PN Avg.	0.09	4.55	0.62	4.14	56.12	8.52	17.60	5.67	1.05	0.98	0.41	0.25	10.49	0.14
C_2-41 PN S.E.	0.00	0.21	0.05	0.24	1.81	0.70	0.71	0.30	0.05	0.03	0.02	0.01	0.51	0.01
C_2-41 PM Avg.	0.00	4.75	0.58	8.06	52.36	9.05	17.08	5.50	1.03	0.93	0.42	0.25	14.51	0.12
C_2-41 PM S.E.	0.00	0.24	0.04	0.50	1.83	0.64	0.67	0.27	0.05	0.03	0.02	0.02	0.81	0.01
P>F	**0.00**	0.56	0.52	**0.00**	0.16	0.58	0.59	0.68	0.75	0.23	0.76	0.82	**0.00**	0.26
WS PN Avg.	0.12	4.31	0.19	2.65	67.32	2.25	16.20	4.62	0.76	1.10	0.29	0.19	8.32	0.04
WS PN S.E.	0.06	0.03	0.02	0.08	0.36	0.21	0.21	0.07	0.02	0.01	0.03	0.02	0.10	0.00
WS PM Avg.	0.00	4.21	0.16	6.54	63.84	2.63	15.67	4.56	0.78	1.05	0.34	0.21	12.09	0.04
WS PM S.E.	0.00	0.10	0.03	0.16	0.28	0.04	0.24	0.07	0.02	0.02	0.01	0.01	0.23	0.01
P>F	**0.07**	0.32	0.29	**0.00**	**0.00**	0.11	0.10	0.53	0.32	**0.03**	0.10	0.25	**0.00**	**0.05**

Table 15. Differences in FA composition (mole%) in *B. napus* transformed with cDNA encoding cat's claw 16:0-ACP desaturase using the HIP extraction followed by methylation with either NaMeth or methanolic HCl-summer 2001 plants.

Once FAMES were obtained they were run on a GLC as described in MATERIALS AND METHODS. Samples included C_2-31A (n=7) and C_2-41 (n=10) and Westar (n=12). ANOVA testing was performed with all significant P-values ($p < 0.07$) shown in **bold**. Abbreviations in the table include H for the HIP, N for NaMeth or M for methanolic-HCl.

	14:0	16:0	16:1 Δ9	18:0	18:1 Δ9	18:1 Δ11	18:2 Δ9,12	18:3 Δ9,12,15	20:0	20:1 Δ11	22:0	24:0	SFA	16:1/16:0
C_2-31A HN Avg.	0.06	4.37	0.46	3.06	62.72	6.13	15.27	5.24	0.93	1.10	0.41	0.25	9.09	0.11
C_2-31A HN S.E.	0.01	0.05	0.01	0.15	0.76	0.34	0.45	0.14	0.03	0.02	0.01	0.01	0.19	0.00
C_2-31A HM Avg.	0.09	4.31	0.44	4.34	61.50	6.30	15.13	5.16	0.94	1.09	0.44	0.26	10.38	0.10
C_2-31A HM S.E.	0.00	0.06	0.01	0.16	0.75	0.27	0.42	0.14	0.03	0.02	0.01	0.01	0.22	0.00
P>F	**0.07**	0.44	0.27	**0.00**	0.28	0.70	0.82	0.68	0.71	0.70	0.17	0.47	**0.00**	0.48
C_2-41 HN Avg.	0.16	4.38	0.62	4.07	56.11	8.88	17.80	5.32	1.04	0.98	0.41	0.22	10.29	0.14
C_2-41 HN S.E.	0.08	0.15	0.05	0.27	1.68	0.71	0.58	0.28	0.06	0.03	0.02	0.02	0.50	0.01
C_2-41 HM Avg.	0.00	4.38	0.57	8.83	52.49	9.03	17.08	5.00	1.04	0.92	0.41	0.23	14.90	0.13
C_2-41 HM S.E.	0.00	0.16	0.05	0.35	1.67	0.72	0.55	0.25	0.06	0.02	0.02	0.01	0.57	0.01
P>F	**0.06**	0.99	0.49	**0.00**	0.14	0.88	0.38	0.39	1.00	0.13	0.85	0.60	**0.00**	0.39
WS HN Avg.	0.07	4.19	0.20	2.51	67.29	2.42	16.33	4.67	0.73	1.10	0.32	0.19	8.01	0.05
WS HN S.E.	0.01	0.03	0.00	0.07	0.25	0.05	0.18	0.08	0.01	0.01	0.01	0.00	0.07	0.00
WS HM Avg.	0.02	4.17	0.11	6.48	64.10	2.47	15.84	4.47	0.75	1.05	0.34	0.20	11.95	0.03
WS HM S.E.	0.01	0.03	0.03	0.43	0.39	0.03	0.18	0.08	0.01	0.01	0.01	0.01	0.43	0.01
P>F	**0.00**	0.56	**0.01**	**0.00**	**0.00**	0.35	**0.06**	0.11	0.30	**0.00**	**0.06**	0.34	**0.00**	**0.01**

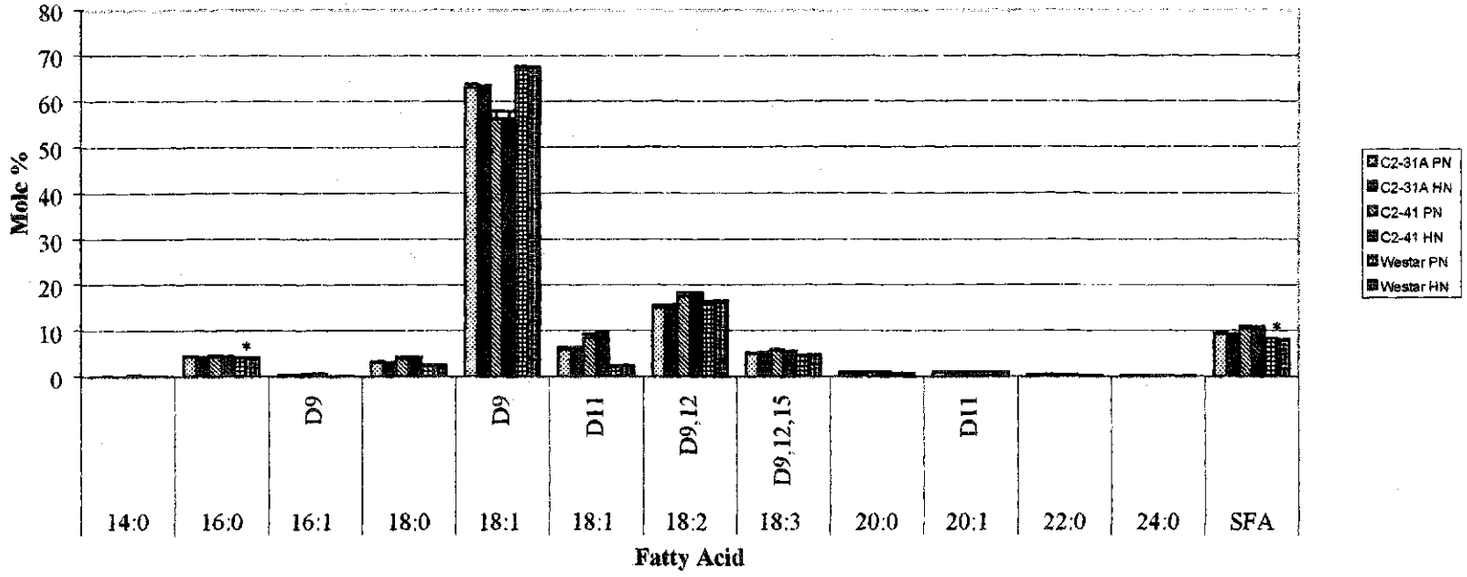

Figure 36. Differences in FA composition (mole) of *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using the PE or HIP extraction and NaMeth methylation-summer 2001 plants . Comparisons were made for *B. napus* containing the cats claw desaturase gene plant line C_2-31A Pe NaMeth (PN) (n=7) and C_2-31A HIP NaMeth (HN)(n=7), C_2-41 PE NaMeth (PN)(n=10) and C_2-41 HIP NaMeth (HN) (n=10), and the untransformed Westar Pe NaMeth (PN) (n=12) and Westar HIP NaMeth (HN) (n=12).

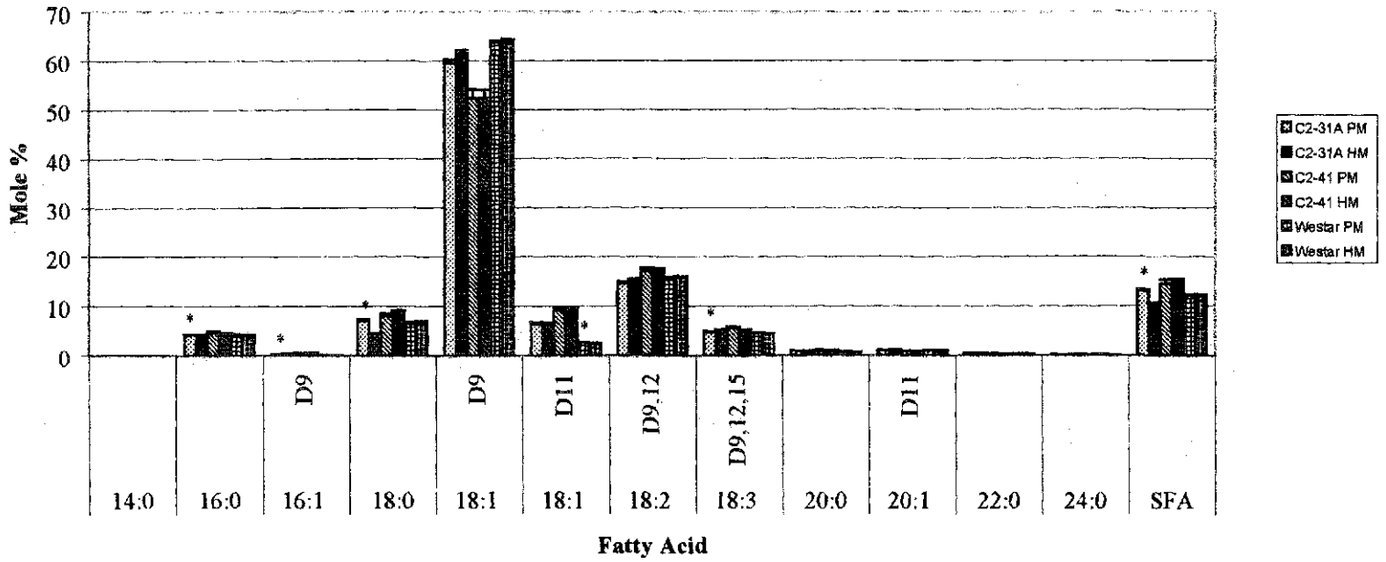

Figure 37. Differences in FA composition (mole%) of *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using the PE or HIP extraction and methanolic-HCl methylation- summer 2001 plants. Comparisons were made for *B. napus* containing the cats claw desaturase gene plant line C_2-31A PE M-HCl (PM) (n=7) and C_2-31A M-HCl (HM)(n=7), C_2-41 PE M-HCl (PM) (n=10) and C_2-41 HIP M-HCl (HM) (n=10), and the untransformed Westar PE M-HCl (PM) (n=12) and Westar HIP M-HCl (HM) (n=12).

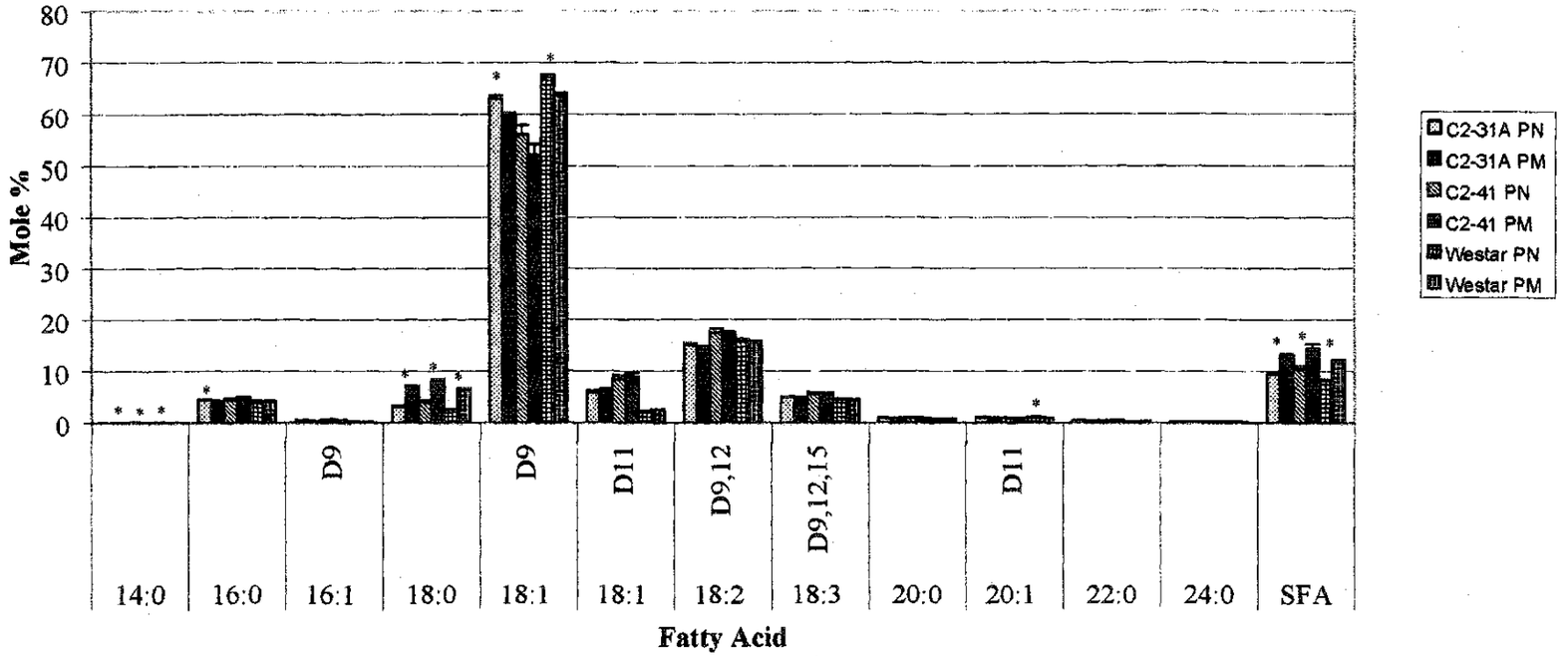

Figure 38. Differences in FA composition (mole%) of *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using the PE extraction and either NaMeth or methanolic-HCl methylation- summer 2001 plants. Comparisons were made for *B. napus* containing the cats claw desaturase gene plant line C_2-31A PE NaMeth (PN) (n=7) and C_2-31A PE M-HCl (PM) (n=7), C_2-41 PE NaMeth (PN) (n=10)and C_2-41 PE M-HCl (PM) (n=10), and the untransformed Westar Pe NaMeth (PN) (n=12) and Westar PE M-HCl (PM) ((n=12).

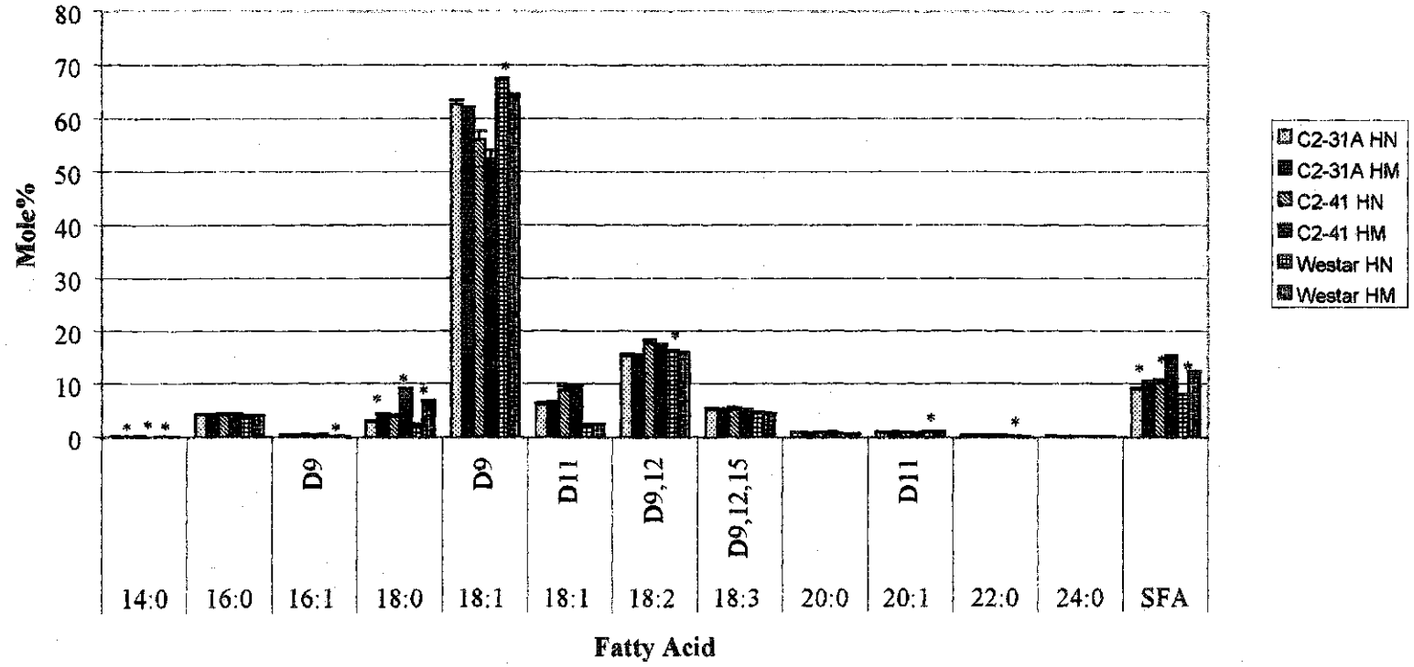

Figure 39. Differences in FA composition (mole%) of *B. napus* transformed with a cDNA encoding cat's claw 16:0-ACP desaturase using the HIP extraction and NaMeth or methanolic-HCl methylation- summer 2001 plants. Comparisons were made for *B. napus* containing the cats claw desaturase gene plant line C_2-31A HIP NaMeth (HN) (n=7) and C_2-31A HIP M-HCl (n=7), C_2-41 HIP NaMeth (HN) (n=10) and C_2-41 HIP M-HCl (HM) (n=10), and the untransformed Westar HIP NaMeth (HN) (n=12) and Westar HIP M-HCl (HM) (n=12).