Effects of Genotype and Environment on the Level of the Target Fatty Acids of Acyl-ACP Thioesterase Transgenes in *Brassica napus* L.

BY
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A Thesis
Submitted to the Faculty of Graduate Studies
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ABSTRACT

Tang, Jihong. PhD, The University of Manitoba, August 30, 2001. Effects of Genotype and Environment on the Level of the Target Fatty Acids of Acyl-ACP Thioesterase Transgenes in *Brassica napus* L. Major Professor: Rachael Scarth.

Canola plants (*Brassica napus* L.) transformed with special acyl-ACP thioesterase (TE) genes accumulate lauric acid (C12:0) or enhanced levels of palmitic acid (C16:0) in the seed oil. Such oils are valuable in manufacturing of a number of products, e.g. margarine, shortening and detergents. There is a need for studies on the level of the target fatty acids of TE and the stability of the expression as influenced by genetic and environmental factors.

The influence of recipient genotype on the target fatty acids was observed in both F_1 seeds and doubled haploid (DH) lines produced from crosses of four transgenic parents carrying the bay-TE (*Uc FatB1*), elm-TE (*Ua FatB1*), nutmeg-TE (*Mf FatB1*) or cuphea-TE (*Ch FatB1*) transgenes with three non-transgenic cultivars possessing distinct fatty acid compositions. Expression of the TE transgenes in low erucic acid (C22:1) genotypes produced significantly higher levels of the target fatty acids than in high C22:1 genotypes. Maternal effects were detected as reciprocal F_1 seeds could be significantly different in the level of the target fatty acids, but cytoplasmic effects were not found since transgenic DH lines from reciprocal crosses showed the same level of the target fatty acids.

Both genomic position and copy number of the TE transgenes could influence the target fatty acids. DH lines carrying one to five copies of the cuphea-TE transgene displayed a positive linear correlation between copy number and the target fatty acid level ($r = 0.77^{**}$). DH lines with the elm-TE transgene at four different loci accumulated significantly different levels of C16:0.

No plants with a completely silenced TE transgene were found by PCR and Southern blotting analyses of more than 300 transgenic DH plants. Most DH lines examined in this study showed a stable level of the target fatty acids over two or three generations tested. However, the level of the target fatty acids was affected by growing conditions. DH lines carrying the elm-TE or the cuphea-TE grown under high temperature conditions (25/20 °C, day/night) showed higher levels of the target fatty acid C16:0 than under lower temperature conditions (20/15 °C).

FOREWORD

This thesis, entitled "Effects of Genotype and Environment on the Level of the Target Fatty Acids of Acyl-ACP Thioesterase Transgenes in *Brassica napus* L.", was written in manuscript style. The three chapters, Chapter 3, 4 and 5, followed the style and format recommended by the journal Crop Science, published by the Crop Science Society of America, Inc., Madison, USA. Part of Chapter 3 was presented at the 1999 American Oil Chemists' Society (AOCS) Annual Conference in Orlando, Florida, and was recognized by a Honored Student Award and a Student Paper Award.

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACCase	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
AP	Alkaline phosphatase
aRNA	Aberrant RNA
asRNA	Antisense RNA
Bay-TE	Acyl-ACP thioesterase encoded by *Uc FatB1* cloned from bay tree
C.V.	Coefficient of variation
C4:0	Butyric acid
C6:0	Caproic acid
C8:0	Capric acid
C10:0	Caprylic acid
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	α-Linolenic acid (9,12,15-octadecatrienoic acid)
C20:0	Arachidic acid
C20:1	Gadoleic acid
C22:0	Behenic acid
C22:1	Erucic acid (13-docasaenoic acid)
CAT	Chloramphenicol acetyl transferase
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide

Cuphea-TE	Acyl-ACP thioesterase encoded by *Ch FatB1* cloned from cuphea
DAGAT	Diacylglycerol acyltransferase
DH	Doubled haploid
DIG	Digoxygenin
DmDM	DNA-mediated methylation
dsRNA	Double-stranded RNA
dsRNase	dsRNA-specific RNase
EFA	Essential fatty acids, i.e. n-3 and n-6 fatty acids
Elm-TE	Acyl-ACP thioesterase encoded by *Ua FatB1* cloned from elm
EMS	Ethyl-methanesulfonate
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
GBSS1	Granule-bound starch synthase 1
GH	Greenhouse
GPAT	Glycerol-3-phosphate acyltransferase
GR	Growth room
GUS	β-glucuronidase, encoded by *uidA*
HDGS	Homology-dependent gene silencing
HEAR	High erucic acid rapeseed
HPT	Hygromycin phosphotransferase
ICR	Intrachromosomal recombination
IR	Inverted repeat
KAR	β-ketoacyl-ACP reductase
KAS	β-ketoacyl-ACP synthase
KCS	β-ketoacyl-CoA synthase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDS	Fisher's least significant difference
LPAAT	Lysophosphatidic acid acyltransferase

MAR	Matrix attachment region
MCFA	Medium chain fatty acids
MGDG	Monogalactosyl diaceylglycerol
MT	Microspore treatment
nptII	Neomycin phosphotransferase gene II from transposon Tn5
Nutmeg-TE	Acyl-ACP thioesterase encoded *by Mf FatB1* cloned from nutmeg
PCR	Polymerase chain reaction
PTGS	Post-transcriptional gene silencing
PUFA	Polyunsaturated fatty acid
QTL	Quantitative trait loci
RCB	Randomized complete block
RdDM	RNA-directed DNA methylation
RdRP	RNA-dependent RNA polymerase
RPS	Repetitive sequence
RT	Root treatment
SP	Self-pollinated
ssRNA	Single-stranded RNA
TAG	Triacylglycerols
TE	Acyl-acyl carrier protein thioesterase
TGS	Transcriptional gene silencing
Ti plasmid	Tumour-inducing plasmid
vir	Virulence genes
VLCFA	Very long chain fatty acids

CHAPTER 1

INTRODUCTION

Canola varieties grown in Canada belong to either the *Brassica napus* or *B. rapa* species which in turn belong to the mustard (*Cruciferae*) family, with *B. napus* occupying more than 80% of the total canola growing acreage (Eskin et al., 1996). The major fatty acids in the seed oil of *B. napus* cultivars include palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and erucic acid (C22:1). C22:1 is present in a large amount in the seed oil of traditional rapeseed cultivars, and is increased to more than 50% by conventional breeding in the seed oil of high erucic acid rapeseed (HEAR) cultivars. Canola quality cultivars are characterized by producing seed oil with less than 2% C22:1 (Eskin et al., 1996; McVetty and Scarth, 2002).

Biosynthesis of the fatty acids begins with the carboxylation of acetyl-CoA to form malonyl-CoA in the plastid (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997; Töpfer et al., 1995). After the malonyl group is transferred to acyl carrier protein (ACP), a polypeptide functionally equivalent in many ways to CoA (Voet and Voet, 1995), malonyl-ACP is condensed with a primer, acetyl-CoA or acetyl-ACP, to form acetoacetyl (C4)-ACP to initiate a cyclic elongation process (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997). The cyclic elongation process, catalysed by fatty acid synthase (FAS), consecutively add two-carbon units (C_2 units) derived from malonyl-ACP to the growing acyl chain of acyl-ACP. In most oil-accumulating plant species, seven cycles of elongation result in the formation of C16:0, with one more cycle to produce C18:0. Subsequent desaturation of C18:0 in the plastid results in C18:1. However, in some plant species, e.g. palm (*Elaeis guineensis* Jacq.), coconut (*Cocos nucifera* L.) and *Cuphea* species, special thioesterases (TE) prematurely terminate the elongation process by hydrolysing the growing acyl chain from ACP, resulting in fatty acids of 8-14 carbons in length being accumulated in the seed oils (Harwood et al. 1994; Ohlrogge and Jaworski, 1997). C18:2 and C18:3 are the products of desaturation of C18:1 in the cytoplasm, and C22:1 is produced from further elongation of C18:1 catalysed by elongase, a

multisubunit complex similar to FAS (Fehling and Mukherjee, 1991; Ohlrogge, 1994). The fatty acids are assembled with glycerol-3-phosphate into triacylglycerols (TAG), the final storage form of the fatty acids in seed (Frentzen, 1993; Töpfer et al., 1995; Voet and Voet, 1995).

The fatty acid composition of the seed oil of oilseed crops is modified to suit various food and non-food applications (Friedt and Lühs, 1998; Murphy, 1994; Töpfer et al., 1995). In addition to canola cultivars with low C22:1 levels and HEAR cultivars, employment of traditional breeding approaches has produced canola cultivars with less than 3% C18:3 and cultivars with low C18:3 and high C18:1 levels in *B. napus* (Scarth and McVetty, 1999).

Advances in biotechnology have provided plant breeders with additional approaches for manipulating the genes coding for the enzymes in the fatty acid biosynthetic pathways to develop cultivars producing speciality oils for particular uses (Murphy, 1994; Somerville, 1993; Töpfer et al., 1995). Genes coding for most of the enzymes in the biosynthetic pathways of the major fatty acids have been cloned from one or another plant species, e.g. the genes of acetyl-CoA carboxylase (ACCase), β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase (KAR), Δ9-, Δ12- and Δ15 desaturases (Harwood and Page, 1994; Napier et al., 1999; Ohlrogge and Jaworski, 1997; Töpfer et al., 1995; Verwoert et al., 1995). As well, genes encoding TE with preference for each of the saturated substrates from C8:0- to C16:0-ACP and for C18:1-ACP have been cloned (Hawkins and Kridl, 1998).

Genetic transformation of *B. napus* plants is commonly achieved by using the *Agrobacterium*-mediated transformation system (Hinchee et al., 1994; Poulsen, 1996). Transgenic plants producing oils with significantly modified fatty acid compositions have been obtained after transformations with a number of genes involved in the fatty acid biosynthetic pathways. These genes include those coding for desaturase (Fader et al., 1995; Hitz et al., 1995; Knutzon et al., 1992), elongase (Töpfer 1995), KAS (Bleibaum et al., 1993; Verwoert et al., 1995), and especially the genes coding for TE cloned from some plant species accumulating high levels of medium chain fatty acids (MCFA), with chain length of 8 to 14 carbon atoms, in the seed oil (Hawkins and Kridl, 1998; Jones et al., 1995,

Martini et al., 1995; Voelker et al., 1992, 1996, 1997; Yuan et al., 1995).

The seed oil of non-transgenic canola cultivars contains only traces of C12:0, ca. 4% C16:0 and 2% C18:0, with the other C18 fatty acids (C18:1, C18:2 and C18:3) accounting for more than 90% of the total fatty acids (Eskin et al., 1996; Voelker et al., 1996). Plants transformed with the bay-TE gene *UcFatB1* cloned from California bay (*Umbellularia californica*), a species accumulating a large amount of C12:0 and C10:0 in the seed (Pollard et al., 1991), produce seed oils with up to 60% C12:0 (Voelker et al., 1996). Plants transformed with the elm-TE (*Ua FatB1*), nutmeg-TE (*Mf FatB1*) or cuphea-TE gene (*Ch FatB1*) cloned from elm (*Ulmus americana*), nutmeg (*Myristica fragranceae*) (Voelker et al., 1997) and cuphea (*Cuhpea hookeriana*) (Jones, et al., 1995), respectively, produce oils with more than 30% C16:0. Oils rich in C12:0 are valuable for uses in the confectionary industry, simulated food products, icing and frostings (Del Vecchio, 1996). High C16:0 oil is also of an economic value. Soybean oil with high levels of C16:0 (23-26%) showed a greater oxidative stability than the normal soybean oil (11% C16:0), thus reducing the need for hydrogenation in producing some food products, such as shortening and margarine (Shen et al., 1997).

Many factors can influence the expression of transgenes. The effects of genetic background, genomic position and copy number of transgenes, as well as growing condition of transgenic plants, on the expression, have been reported (Charrier et al., 2000; Dale et al., 1998; Maqbool and Christou, 1999). There is little information about the influence of these factors on the expression of TE transgenes. A better understanding of the expression and stability as influenced by these factors would facilitate the breeding and commercial production of TE transgenic cultivars.

The influence of recipient genotype on the expression of transgenes has been reported in a number of plant species (Blundy et al., 1991; Scott et al., 1998; Xu et al., 1997). For example, a study with a white clover line (*Trifolium repens* L.) carrying an intact copy of the β-glucuronidase (GUS) gene showed a four-fold variation in the GUS activity between plants even though each plant contained a copy of the same T-DNA insert (Scott et al., 1998). Progeny from plants with high levels of the

expression showed higher GUS activity in the next generation than those from plants with lower expression, indicating the inheritance of a genetic influence from the recipient genotype on the transgene expression.

Comparison between the fatty acid composition of the seed oil of TE transgenic plants and the fatty acid composition of the original plant species from where the TE had been cloned suggests an influence of recipient genotype on transgene expression. The levels of the fatty acids targeted by TE in *B. napus* transgenic lines are usually lower than found in the original plant species of the TE (Dehesh et al., 1996; Hawkins and Kridl, 1998; Jones et al., 1995; Voelker et al. 1997). For example, in the seed oil of California bay tree (*U. californica*), the total level of C12:0 and C10:0 can be up to 70% (Pollard et al., 1991; Töpfer et al., 1995), while plants transformed with the bay-TE gene (*Uc FatB1*) had less than 60% C12:0 and traces of C10:0 (Voelker et al., 1996). The lower levels of the target fatty acids in transgenic plants could be due to differential activity and substrate specificity of some enzymes involved in the plant fatty acid biosynthetic pathways in the *B. napus* genetic background, e.g. β-ketoacyl ACP synthase (KAS), which catalyzes the elongation of fatty acyl chain-ACP, and acyl-ACP acyltransferase, which assembles fatty acids into triacylglycerols (Dehesh et al., 1996; Hawkins and Kridl, 1998; Knutzon et al., 1992; Voelker et al., 1997). However, it has not been determined whether different genotypes within *B. napus* could differently affect the production of the target fatty acids.

The effects of genomic position and copy number of transgenes on the expression level have been widely reported in plants (Allen et al., 2000; De Neve et al., 1999; Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999). Since foreign DNA integrates almost exclusively at random non-homologous sites during the transformation process, some integrations may occur in transcriptionally active chromatin environments, others in condensed, transcriptionally inert chromatin regions (Allen et al., 2000; Mengiste and Paszkowski, 1999). It is believed that transgenes in heterochromatic areas such as those surrounding centromeres are prone to silencing and give rise to reduced and/or variable expression (Allen et al., 2000; Maqbool and Christou, 1999; Mengiste and Paszkowski, 1999; Weiler and Wakimoto, 1995). Literature about the relationship

between copy number and expression level is conflicting (Hobbs et al., 1993; McCabe et al., 1999). The two variables were found to be negatively correlated (Cervera et al., 2000; Hobbs et al., 1993; Mannerlöf et al., 1997), not correlated (Bauer et al., 1998; Hobbs et al., 1993; McCabe et al., 1999), or positively correlated (Gendloff et al., 1990; Hobbs et al., 1993; van der Hoeven et al., 1994).

For practical applications, it is important that transgenes are inherited and expressed in a predictable, consistent, and stable manner (Campbell et al., 2000; Conner and Christey, 1994). Voelker et al. (1996) reported that *B. napus* lines with multiple copies (5-15) of the bay-TE transgene (*Uc FatB1*) were stable for more than five generations, without any apparent genetic instability and loss of transgenic phenotype. However, instability in the expression has been observed for some transgenes in some plant species (Assaad et al., 1993; Scheid et al., 1991; Zhong et al., 1999). In a population of *Arabidopsis thaliana* plants transgenic for a hygromycin resistance gene (*hpt*), 50% of the plants failed to transmit the resistant trait to the progeny although the complete transgene was detected in all the plants (Scheid et al., 1991).

Unstable expression of transgenes has been associated with gene silencing as gene silencing is a reversible process (Charrier et al., 2000; Meyer and Saedler 1996; Scott et al., 1998). Gene silencing is defined as somatically or meiotically heritable repression of gene expression that is potentially reversible and is not due to mutation (Kaeppler et al., 2000). Silencing could result from the blocking of transcription initiation, i.e. transcriptional gene silencing (TGS), or from the degradation of mRNA after transcription, i.e. post-transcriptional gene silencing (PTGS) (Chandler and Vaucheret, 2001; Matzke and Matzke 1998; Wassenegger, 2000). Possible factors inducing gene silencing include multiple copies, special structure of transgene inserts (e.g. tandem repeats and truncated copies), vector sequence, special genomic site, as well as in vitro culture and growing conditions (Charrier et al., 2000; Dale et al., 1998; Maqbool and Christou, 1999). The stability of the expression of TE transgenes in different growing conditions has not been investigated previously.

The objectives of this study were:

1) To examine the effects of (nuclear) recipient genotype, cytoplasm, and maternal plant on the

expression of TE transgenes;

2) To test the effects of genomic position and copy number of TE transgenes on the expression;

3) To assess the stability of TE transgenic DH lines over generations and under different growing conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosynthesis of Plant Fatty Acids and Triacylglycerols

The fatty acids commonly found in the seed oil of major oilseed crops, e.g. rapeseed, soybean, oil palm, cotton seed and groundnut, include palmitic acid (C16:0), stearic acid (C18:0) and their desaturated products - oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids (McVetty and Scarth, 2002; Töpfer et al., 1995). These fatty acids are the most important nutritionally and functionally as they account for over 90% of the fatty acids in the average American diet (Groff and Gropper, 2000). The fatty acids having a chain length of 16 and 18 carbon atoms are known as long-chain fatty acids; C8:0 to C14:0 are referred to as medium-chain fatty acids (MCFA) (Dehesh et al., 1996; van de Loo et al., 1992; Voelker et al., 1992, 1997). The fatty acid profile is synonymous to the total composition of fatty acids in the triacylglycerols (TAG) of the seed oil (McVetty and Scarth, 2002).

2.1.1 The Biosynthetic Pathway

Fatty acid biosynthesis occurs exclusively in the stroma of the plastid, unlike the modifications of fatty acyl residues and TAG assembly which take place in the cytoplasm except for the plastidial desaturation (Töpfer et al., 1995). The synthetic process starts from the carboxylation of acetyl-CoA to form malonyl-CoA. After the malonyl group is transferred to acyl carrier protein (ACP), a polypeptide functionally equivalent in many ways to CoA (Voet and Voet, 1995), malonyl-ACP is condensed with a primer, acetyl-CoA or acetyl-ACP, to form acetoacetyl (C4)-ACP to initiate a cyclic process (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997). The cyclic process, catalysed by fatty acid synthase (FAS), consecutively add two-carbon units (C_2 units) derived from malonyl-ACP to the growing acyl chain of acyl-ACP. Each cycle consists of: 1) a condensation reaction catalysed by β-ketoacyl-ACP synthase (KAS), 2) a reduction reaction catalysed by β-hydroxyacyl-ACP reductase, 3) a dehydration reaction due to the function of β-hydroxyl-ACP dehydratase, and 4) another reduction due to enoyl-ACP dehydratase. In most oil-accumulating plant

species, seven cycles of elongation lead to the production of C16:0, with one more cycle to C18:0, and subsequent desaturation of C18:0 in the plastid resulting in C18:1. However, in some species, e.g. oil palm (*Elaeis guineensis* Jacq.), coconut (*Cocos nucifera* L.), and *Cuphea* species, special thioesterases prematurely terminate the elongation process by hydrolysing the growing acyl chain from ACP, resulting in the accumulation of MCFA in the seed oil (Harwood and Page, 1994).

Of the newly synthesized fatty acyl chains (C16:0-, C18:0- and 18:1-ACP), only a small portion are transferred from ACP to glycerol-3-phosphate for membrane lipid synthesis within the plastid (the prokaryotic pathway) (Jones et al., 1995; Ohlrogge and Jaworski, 1997); most are released from ACP by the action of thioesterases. The free fatty acids cross the plastid envelope membrane (Jones et al., 1995; Ohlrogge, 1994; Ohlrogge and Jaworski, 1997), after which they are re-esterified to CoA by acyl-CoA synthase located in the outer envelope of the plastid (Töpfer et al., 1995). In the cytoplasm, some C18:1-CoA undergo modifications, including desaturation to introduce additional double bonds or further elongation which occurs in C22:1 producing species (Ohlrogge, 1994).

The assembly of fatty acids with glycerol-3-phosphate into TAG is a process of stepwise acylation of glycerol-3-phosphate taking place in the endoplasmic reticulum (ER). The first step is catalysed by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid with a fatty acyl chain being incorporated into the *sn*-1 position of the glycerol backbone. Lysophosphatidic acid is converted to TAG by successive actions of lysophosphatidic acid acyltransferase (LPAAT), phosphatase (PA) and diacylglycerol acyltransferase (DAGAT) (Frentzen, 1993; Töpfer et al., 1995; Voet and Voet, 1995). LPAAT and DAGAT are responsible for the incorporations of fatty acids into the *sn*-2 and *sn*-3 positions of the glycerol backbone, respectively. All the three acyltransferases are substrate specific; e.g., the LPAAT of *Brassica* is more active on unsaturated C18-CoA with a stronger preference to C18:2 than C18:1(Frentzen, 1993; Töpfer et al., 1995).

2.1.2 Major Enzymes

Acetyl-CoA carboxylase (ACCase): ACCase catalyses the first committed step of fatty acid synthesis, the formation of malonyl-CoA from acetyl-CoA, to provide the essential substrate for FAS

(Harwood and Page, 1994; Voet and Voet, 1995). Plants have the both forms of ACCase found in nature. Homodimeric multifunctional ACCase of plants is similar to that of animal and yeast and is probably localized in the cytosol. Plant heteromeric ACCase has at least four subunits as found in bacteria and is localized in the plastid (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997). It is the heteromeric plastid form that catalyses the formation of malonyl-ACP in the plastid (Ohlrogge and Jaworski, 1997). The β-carboxyltransferase subunit of plastid ACCase is plastome encoded; the other three subunits, biotin carboxylase, biotin carboxyl carrier protein and α-subunit of carboxyltransferase, are nuclear encoded (Ohlrogge and Jaworski, 1997). ACCase is a primary determinant of the fatty acid synthesis rate (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997; Töpfer et al., 1995).

Fatty acid synthase (FAS): The function of FAS is to transfer the malonyl moiety from malonyl-CoA, formed by the action of ACCase, to ACP, and to elongate acyl-ACP with malonyl-ACP as the C_2 carbon unit donor (Ohlrogge and Jaworski, 1997). Plant FAS, like that in bacteria, is classified as Type II synthase, consisting of individual proteins that can be isolated in an active enzyme form (Harwood and Page, 1994; Ohlrogge et al., 1993; Verwoert et al., 1995). The FAS complex include: 1) acetyl-CoA:ACP transacylase (AAT), which is responsible for the formation of acetyl-ACP; 2) malonyl-CoA:ACP transacylase (MCAT), which transfers the malonyl group from CoA to ACP; 3) β-ketoacyl-ACP synthase (KAS) (Harwood and Page, 1994; Verwoert et al., 1995), including isoforms KAS I, II, III and IV; 4) β-ketoacyl-ACP reductase (KAR); 5) β-hydroxyacyl-ACP dehydratase; and 6) enoyl-ACP reductase (Dehesh et al., 1996; Harwood and Page, 1994; Verwoert et al., 1995).

Of the KAS isoforms, KAS III catalyses the first condensation reaction - the condensation of malonyl-ACP with a primer. The primer which KAS III prefers is acetyl-CoA rather than acetyl-ACP. Thus, KAS III may play a regulatory role in the total fatty acid synthesis (Harwood and Page, 1994; Ohlrogge and Jaworski, 1997; Verwoert et al., 1995). KAS I is responsible for the elongation of the acyl chain up to C16, and KAS II extends C16:0 to C18:0 (Harwood and Page, 1994;Verwoert et al., 1995). The relative proportion of C16 : C18 fatty acids in the seed oil of oilseed crops could

be regulated by the activity of KAS II in relation to KAS I and III (Harwood and Page, 1994). KAS IV, identified later from in *Cuphea*, is specialized for the formation of MCFA; its co-expression with a medium-chain TE transgene strongly enhances the level of MCFA in the seed oil than the TE alone in transgenic *B. napus* plants (Dehesh et al., 1996). In addition, Slabaugh et al. (1998) cloned a KAS isoform with a similar specificity to KAS IV.

Desaturase: Desaturation of a fatty acid involves the enzymatic removal of hydrogen from a methylene group in the acyl chain, which is a highly energy-demanding reaction requiring an activated oxygen intermediate (Shanklin and Cahoon, 1998). Desaturation of C18:0 is catalysed by Δ9-desaturase localized in the stroma of the plastid, which adds a double bond between carbon atoms Δ9 and Δ10, counted from the carboxyl end of C18:0. In oilseed crops, Δ9-desaturase converts most C18:0-ACP synthesized in the plastid to C18:1-ACP (Töpfer et al., 1995). The higher preference of Δ9-desaturase for C18:0-ACP over C16:0-ACP, could be the explanation for the higher percentage of C16:0 than C16:1, and C18:1 than C18:0, in the seed oil of most oilseed crops (Harwood and Page, 1994). Once C18:0 leaves the plastid, it is not further desaturated since higher plants, unlike animals and yeast, do not contain Δ9-desaturase in the ER (Somerville, 1993). The additions of double bonds to C18:1 take place in the cytosol, and are catalysed consecutively by Δ12- and Δ15-desaturases, leading to the production of C18:2 and C18:3 (Harwood and Page, 1994; Heinz, 1993; Töpfer et al., 1995). Genes encoding desaturases have been cloned from a number of plant species (Napier et al., 1999). In the *B. napus* genome, there are four to six gene copies coding for Δ12-desaturase and six to eight copies for Δ15-desaturase (Scheffler et al., 1997). The multigene families arose through genome duplication. Before the two progenitor species, *B.oleracea* and *B. rapa*, combined to form *B. napus*, the genes of the desaturases were at least duplicated or triplicated (Scheffler et al., 1997).

Acyl-ACP thioesterase (TE): TEs specific for medium-chain acyl groups have been detected in more than ten plant species since Pollard and co-workers (1991) first provided biochemical evidence for TE (Jones et al., 1995; Töpfer et al.1995; Yuan et al., 1995). TEs are also known as hydrolases, and they can hydrolyse the thioester bond between the acyl group and ACP, thus terminating the acyl

chain elongation process (Jones et al., 1995; Töpfer et al., 1995; Yuan et al., 1995). TEs are substrate specific; they are strict for the length of the acyl chain in the acyl-ACP substrates (Dehesh et al., 1996; Voelker and Davies, 1994). The commonly identified plant TE, C18:1-TE, is ubiquitous and has a preference for C18:1-ACP, with a limited activity on C16:0- and C18:0-ACP. The subsequently identified C16:0-TE, active on C14:0- to C18:0-ACP with a strong preference for C16:0, is also ubiquitous, and it is not restricted to MCFA-producing plant species (Hawkins and Kridl, 1998; Jones et al., 1995).

Genes coding for TEs with preference for each of the saturated substrates from C8:0- to C16:0-ACP, as well as for C18:1-ACP, have been cloned, with the exception of C18:0-ACP (Hawkins and Kridl, 1998). Based on the amino acid sequence homology, TEs from higher plants are classified into two distinct classes: FatA and FatB TEs (Jones, et al., 1995). C18:1-TE is the only member identified in the FatA class; it is nuclear-encoded, synthesized as preprotein in the cytoplasm, and subsequently exported into the plastid (Jones, et al., 1995). In contrast, FatB TEs prefer acyl-ACP with a saturated acyl group, and contain all known plant medium-chain TEs, and C16:0-TE (Jones et al., 1995). C16:0-TE is believed to be the oldest form; other FatB TE and FatA TE are evolved independently from it (Jones et al., 1995).

Elongase: Elongase catalyses the formation of C22:1, which is present in high percentages in seed oils of traditional rapeseed cultivars and related *Brassica* species. This enzyme is likely to be a multisubunit complex, similar to FAS, and have four enzymatic activities (Fehling and Mukherjee, 1991, Töpfer et al., 1995). Elongase uses C18:1-CoA as the substrate and malonyl-CoA as the C_2 unit donor to catalyse the extension of the C18:1 acyl chain via C20:1-CoA to produce C22:1 (Créach et al., 1995; Harwood and Page, 1994; van de Loo et al., 1992). Genes coding for the condensing enzyme, β-ketoacyl-CoA synthase (KCS), of the elongase have been cloned from a few *Brassica* species (Töpfer et al., 1995; Venkateswari et al., 1999).

Acyltransferases: Acyltransferases are responsible for the assembly of fatty acids with glycerol to produce storage and membrane lipids (Voet and Voet, 1995). The three acyltransferases involved

in TAG biosynthesis in the cytoplasm are substrate specific (Frentzen, 1993). In general, GPAT prefers saturated acyl-CoA. *Cuphea* GPAT prefers C12:0-CoA; *B. napus* GPAT is more active on C16:0 and C18:1, whereas that of *B. rapa* excludes C16:0. LPAAT exhibits a stronger specificity than GPAT. LPAAT of *Brassica* is more active on unsaturated C18-CoA with a stronger preference to C18:2 than to C18:1; it is incapable of incorporating saturated fatty acids and C22:1 into the *sn*-2 position in the glycerol backbone (Frentzen, 1993; Harwood and Page, 1994; Töpfer et al., 1995). DAGAT generally has a broader range of specificity; but in some species, e.g. sunflower (*Helianthus annuus* L.), DAGAT prefers C16:0 and C18:1 but not C18:0 (Frentzen, 1993).

2.2 Breeding of Fatty Acid Composition

Fatty acid profiles of oilseed crops are modified to suit various food and non-food applications (Friedt and Lühs, 1998; Murphy, 1994; Töpfer et al., 1995). In most of the major oilseed crops, cultivars producing seed oils with modified fatty acid compositions have been developed using traditional breeding methods and biotechnology-based methods (McVetty and Scarth, 2002).

2.2.1 Erucic Acid

Traditional rapeseed oil contains about 45% erucic acid (C22:1) in its total fatty acids. Feeding high C22:1 oil to several animal species, e.g. rats, rabbits, hamster, monkeys and gerbil, was associated with the mycrocardial damage characterized by fatty deposits around the heart and kidneys and muscle lesions in the heart (Eskin et al., 1996; Taylor et al., 1994). Plants with low levels of C22:1 in the seed oil were identified from a German spring-type *B. napus* forage cultivar 'Liho' in 1959 (Stefansson et al., 1961). The low C22:1 plants were backcrossed with adapted cultivars, resulting in the development of the first low C22:1 *B. napus* cultivar 'Oro' in 1968 and the first low C22:1 *B. rapa* cultivar 'Span' in 1971 (Stefansson and Downey, 1995). By 1974, 95% of the rapeseed growing in Canada was of a low C22:1 fatty acid composition (Eskin et al., 1996). All the canola cultivars registered in Canada must have less than 1% C22:1 in the seed oil, combined with less than 30 mmoles/g glucosinolates in the air-dried oil-free meal (The Canola Council of Canada, 2000; Eskin et al., 1996).

High C22:1 oils and C22:1 derivatives have more than 200 potential or patented industrial applications, e.g. as an additive in lubricants and solvents, as a softener in textiles, and the amide derivative is used in the manufacture of polymers, high temperature fluidity lubricants, surfactants, plasticizers, surface coatings and pharmaceuticals (McVetty and Scarth, 2002; Scarth et al., 1992; Taylor et al., 1994; Töpfer et al., 1995). The cost of purifying C22:1 from *B. napus* oil makes this source less economic than alternative products from petroleum. If the level of C22:1 was increased from around 50%, the level of current HEAR cultivars, to >90%, the cost of C22:1 could fall substantially and the market size would increase significantly (Ohlrogge, 1994; Töpfer et al., 1995).

Plants with high levels of C22:1 were identified from a Swedish *B. napus* summer rape strain after four years' consecutive selections. The first HEAR cultivar with a low glucosinolate content, 'Hero',was released in 1989. It was developed by crossing the high C22:1 plants as the female parent with an adapted high C22:1 rapeseed cultivar 'Reston'(with a high glucosinolate content), followed by selection for improved agronomic performance (Scarth et al., 1991, 1992). Using the same selection procedure, another HEAR cultivar 'Mercury' with 54% C22:1 was developed and registered in 1992 (Scarth et al., 1995a). Recently released HEAR cultivars, 'Castor' and 'MilleniUM01', can produce up to 55% C22:1 (McVetty et al., 1998, 1999). In comparison with the fatty acid composition of canola quality oil, the high C22:1 level of HEAR cultivars, including Mercury, is accompanied by significant decreases in C18:1 and C18:2, with minor changes in the levels of C16:0, C18:0 and C18:3 (Scarth et al., 1995a; McVetty et al., 1998, 1999). No *Brassica* germplasm with > 66% erucic acid has been reported (McVetty and Scarth, 2002). An upper limit for the C22:1 level in *B. napus* has been associated with the substrate specificity of *Brassica* acyltransferase LPAAT, which prefers unsaturated C18 fatty acids and discriminates against very long chain fatty acids (VLCFA), including C22:1. This restricts the C22:1 level in rapeseed oil to a theoretical maximum of 66.7% (Frentzen, 1993; Taylor et al., 1994; Lühs et al.,1999).

Two genetic loci control the C22:1 level in the seed oil of *B. napus* (Chen and Beversdorf, 1990; Downey and Harvey 1963; Harvey and Downey, 1964). HEAR varieties possess readily detectable C18:1-CoA elongase, whereas low C22:1 cultivars do not (Pollard and Stumpf, 1980). The

alleles/genes at the two loci may encode the β-ketoacyl-CoA synthase (KCS) of the elongase complex, which is required for C22:1 synthesis with C18:1-CoA as the substrate (Barret et al.,1998; Fourmann et al., 1998; Han et al., 1998; Lassner et al. 1996; Roscoe et al., 1998). Polymorphism of the KCS gene, originated from minor alterations in the nucleotide sequence (Clemens and Kunst, 1997; Fourmann et al., 1998), has been associated with variation of the C22:1 level in *Brassica* species (Lühs et al., 1999).

2.2.2 Linolenic Acid

Linolenic (C18:3) and linoleic acid (C18:2), together with their longer-chain and more unsaturated derivatives, are the two series of essential fatty acids (EFA), n-3 and n-6 fatty acids, respectively, for human development and health (James et al., 2000; Hornstra, 2000). The two parent EFA, C18:3 and C18:2, cannot be synthesized in vertebrates (James et al., 2000), and are supplied primarily by foods of plant origin (Sanders, 1999). These fatty acids play important roles in reproductive, retinal, and brain functions in the human body (Sanders, 1999; Hornstra, 2000). However, a high C18:3 level in seed oils is associated with poor oxidative and flavor stability, and generation of undesirable room odor during frying (Kochhar, 2000; Rattray et al., 1984; Scarth, 1995). Normally, less stable oils are hydrogenated to enhance the stability, but the hydrogenation process causes formation of a large amount of trans fatty acids and positional isomer fatty acids (Kochhar, 2000). Trans fatty acids contain at least one double bond in the trans configuration. Physiological functions of trans fatty acids, especially their possible roles in atherosclerosis, increase of blood cholesterol, and coronary heart diseases, are of concern although this issue is still subject to controversy (Hayakawa et al., 2000; Lichtenstei, 2000; Nicolosi and Rogers, 1997). In addition, hydrogenation, or addition of natural antioxidative components (e.g. tocopherols) as an alternative to improve the stability, increases the cost of commodity oils (Kochhar, 2000; Ohlrogge, 1994).

Low Linolenic Canola: Breeding for low C18:3 canola cultivars has taken advantage of natural mutation and induced mutagenesis, as well as genetic transformation (Scarth, 1995). *B. napus* germplasm accessions have a variation of 3.3-13.1% in the C18:3 level, and selection for low levels of C18:3 from natural variation is effective (Ishida et al., 1995; Laakso et al., 1995). The first low

C18:3 canola cultivar 'Stellar' was released in 1987 (Scarth et al., 1988, 1992). Its parents included the mutant line 'M11', produced by seed mutagenesis treatment of a Canadian spring-type *B. napus* canola cultivar'Oro'(Röbbelen and Nitsch, 1975). Further selection resulted in the development of 'Apollo' (Scarth et al., 1995a), which has a distinct fatty acid profile with lower C18:3 (1.7%), higher C18:1(67%), and minor changes in other fatty acids - 3.5% C16:0, 2.0% C18:0, 23.7% C18:2, and < 1% C22:1 (Scarth et al., 1995a), in comparison with the typical canola oil fatty acid profile: 8.6% C18:3, 59.7% C18:1, 23.3% C18:2, 3.9% C16:0, 1.15% C18:0 and < 1% C22:1 (McVetty and Scarth, 2002). The low C18:3 trait is controlled by two major recessive genes with additive effect, which block the desaturation pathway (Scarth, 1995; Scarth and McVetty, 2000). Minor genes, maternal and cytoplasmic effects have also been associated with the variation in the C18:3 level in *B. napus* (Bartkowiak-Broda and Krzymanski, 1983; Diepenbrock and Wilson, 1987; Jourdren et al., 1996; Pleines and Friedt, 1989).

Low Linolenic Flax: Linseed (flax, *Linum usitatissimum*), traditionally grown for industrial uses, has more than 45% C18:3 in the seed oil (Green and Marshall, 1984; Rowland, 1992). Seed mutagenesis treatment of a high C18:3 cultivar 'Glenelg' with 0.4% ethyl-methanesulfonate (EMS), followed by half-seed tests of M1 seeds, led to the development of two mutant lines that had about 6-9% less C18:3 than the parent, with a different gene mutated in each line (Green and Marshall 1984; Green, 1986b; Rowland, 1992). Crossing of the two mutants resulted in plants with less than 2% C18:3, and a reduced level of C18:2, with almost unchanged levels of the other fatty acids (Green, 1986a; Rowland, 1992; Rowland and Bhatty, 1990). The first low C18:3 linseed cultivar 'Linola™ '947' was released in Canada in 1993 (Dribnenki and Green, 1995). Its oil contains 2.4% C18:3 and 72% C18:1 in the fatty acid composition, compared to 54% C18:3 and 20% C18:2 in a traditional cultivar used as the parent in the breeding program (Dribnenki and Green, 1995). 'Solin' as a trade name has been registered for linseed oils containing less than 5% C18:3 (Fitzpatrick and Scarth, 1998).

Low Linolenic Soybean: Linolenic acid (C18:3) is also a concern for the seed oil of soybean (*Glycine max* (L.) Merr.). Soybean seed has about 20% oil; C18:3 comprises 5-18% of the total fatty

acids, with an average of about 8-9% (Pantalone et al., 1997; Rattray et al., 1984). Reducing the C18:3 level, together with reducing the level of saturated fatty acids and increasing the C18:1 level, has been set as an important objective in soybean fatty acid breeding (Pro Farmer, 2000b; Wilson, 1996). Germplasm containing only 3.3% C18:3 in the seed oil has been developed by combining two recessive alleles which reduce the activities of Δ12- and Δ15-desaturases, respectively. The low C18:3 trait has been combined with the low C16:0 trait in soybean breeding lines (Wilson, 1996).

2.2.3 Oleic Acid

High Oleic Canola: Unsaturated vegetable oils, such as corn, soybean and canola oil, have been reported to be hypocholesterolemic by virtue of their predominant levels of either C18:2 or C18:1. These fatty acids have an ability to upregulate low density lipoprotein (LDL) receptor activity and/or decrease LDL cholesterol production rate (Nicolosi and Rogers, 1997). Comparative studies showed that oils with high C18:1 and low C18:3 levels possess a higher oxidative stability without extensive hydrogenation, and produce less undesirable products during deep frying (Fitzpatrick and Scarth, 1998; Warner and Mounts, 1993). Concomitant reduction in the contents of polyunsaturated fatty acids (PUFA) could remove the need for partial hydrogenation, thus eliminating the concern with trans fatty acids (Töpfer et al., 1995). Moreover, for oleochemical applications, an increase in the C18:1level to over 90% in seed oils would be of a considerable value because of reduced costs for homogenous or near-homogeneous starting materials (Töpfer et al., 1995).

Regular canola cultivars have about 61% C18:1 in the seed oil (Scarth and McVetty, 1999). Identification of plants with 69% C18:1 in the seed oil, followed by self-pollination and recurrent selection, has led to the development of *B. napus* breeding lines with 85-90% C18:1 (Vilkki and Tanhuanpää, 1995). Mutagenesis treatment of seeds or microspores has resulted in *B. napus* lines producing seed oils with 80-86% C18:1 (Rücker and Röbbelen, 1995; Schierholt and Becker, 1999). *B. napus* cultivars with 70-75% C18:1 and reduced C18:3 level have been released, e.g. 'Clear Valley 75' and 'MONOLA' (Scarth and McVetty, 1999).

High Oleic Sunflower and Soybean: The most dramatic increase in the C18:1 level has been

achieved in sunflower breeding. Regular sunflower cultivars have approximately 20% C18:1 and 11% C18:0 and C16:0 in the seed oil. The high C18:1 and low saturate sunflower cultivar 'SunolaT' developed by using a high C18:1 germplasm from the USSR (the former Soviet Union) produces 89% C18:1 and less than 6% saturates (Fitzpatrick and Scarth, 1998). Sunflower hybrids with up to 90% of C18:1 in the seed oil have been developed by mutation breeding (Cole et al., 1998; Fernández-Martinez et al., 1989). In soybean, a cultivar with 80-85% C18:1, compared to about 23% in traditional cultivars, was planted on small-scale in 1997 (Inform, 1998).

2.2.4 Saturated Fatty Acids

Lauric (C12:0), myristic (C14:0) and stearic (C16:0) acids have been widely reported to raise plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) levels in animals and humans, presumably by decreasing LDL receptor activity and/or increasing LDL-C production. C8:0 and C10:0 also showed similar adverse effects in later studies (Nicolosi and Rogers, 1997). Canola oil is the only vegetable oil to meet the criteria of the low saturated oils (<7%) defined in the labeling regulations of the United States and Canada (Scarth and McVetty, 1999).

Breeding for low levels of saturated fatty acids in soybean has been predominantly achieved through traditional methods (Wilson, 1996). Regular soybean oil has around 11% C16:0 and 4% 18:0 (Downey, 1983; Kinoshita et al., 1998; Wilcox et al., 1994). Soybean mutants with low levels of C16:0, controlled by single recessive alleles, fap1, fap3 or sop1, are identified from germplasm or after mutagenesis treatment (Kinoshita et al., 1998; Wilcox and Cavins, 1990; Wilcox et al., 1994). The mutagenesis-produced fab1 allele affects the activity of a TE with a broad substrate specificity. The fap3 allele, identified from a recurrent selection program with parents originating from Northern China, reduces the activity of C16:0-TE (Wilcox et al., 1994; ARS/USDA, 2000). These genes showed highly heritable additive effects on the C16:0 level. A widely used double homozygous recessive genotype (fap1fap1fap3fap3) produces a seed oil with only 3.7% C16:0 and 2.9% C18:0 (Wilcox et al., 1994; Wilson, 1996). The combination of fap1 with sop1 led to another germplasm with 3.5% C16:0 (Kinoshita et al., 1998). Genetic studies using reciprocal crosses showed no maternal effect and cytoplasmic effect on the C16:0 level and C18:0 level (Kinoshita et al., 1998;

Rahman et al. 1997). The decrease in C16:0 is accompanied by a significant increase in C18:1 and reduction in C18:3, with no significant change in C18:2 (Rebetzke et al., 1998). Small-scale production started in 1999 and low saturated fatty acid and low linoleic acid oil is under further testing for functionality, stability and flavor to ensure that it meets the industrial needs in the U. S. (Pro Farmer editors, 2000a, b).

Replacement of *B. rapa* cultivars with *B. napus* varieties of a relatively higher C16:0 level has resulted in a higher level of saturated fatty acids in commodity canola oil. In order to keep canola competitive, breeding for less than 4% of saturated fatty acids (C16:0 + C18:0) has been initiated with application of conventional methods such as interspecific hybridization, artificial re-synthesis of *B. napus*, and microspore induced mutagenesis (McVetty and Scarth, 2002; Scarth and McVetty, 1999). As a long term priority, breeding for canola cultivars with less than 3.5% saturated fatty acids is encouraged (The Canola Council of Canada, 2000).

Oils with high levels of saturated fatty acids also have applications in the production of margarines and shortenings. For margarine and shortening productions, the melting point of most vegetable oils should be increased, usually by catalytic hydrogenation - a process which increases the saturation level but this also increases costs and produces trans fatty acids (Ohlrogge, 1994; Shen et al., 1997). Breeding for high saturate oils will overcome these problems. Trans-free margarine made from highly saturated (HS) soybean oil was not significantly different in sensory properties from the commercial soft-tub margarine (Kok et al., 1999). In soybean, germplasm with 18% C16:0 has been developed by EMS treatment, and the mutant has a recessive allele fap2 affecting the activity of KAS II (Wilcox and Cavins, 1990). Another mutant with 28.1% C18:0 was identified in progeny derived from crosses involving a Chinese germplasm (Hammond and Fehr, 1983; ARS/USDA, 2000). The high C18:0 level in this mutant is determined by a recessive allele probably affecting the activity of C18:0-ACP desaturase (Hammond and Fehr, 1983; Wilson, 1996). However, plants with the two recessive alleles exhibited only mid-parental levels of C18:0 and C16:0 since fab2 restricts C18:0 synthesis (Wilson, 1996). High C18:0 soybean mutants have also obtained after X-ray irradiation of seeds (Rahman, et al., 1997). In sunflower, three induced mutations increased C18:0 content from

5% to 10-26% (Osorio et al., 1995).

2.3 Factors Influencing the Fatty Acid Composition

2.3.1 Embryo Genotype

In *Brassica*, the seed embryo constitutes approximately 85% of the seed weight, with the remainder being the seed coat (Thomas and Kondra, 1973). Since the oil is synthesized within the embryo cells (Thomas and Kondra, 1973) and there is no evidence that fatty acid molecules from leaf or pod, i.e. maternal tissue, is transmitted to the seed (Pleines and Friedt, 1989), one would anticipate that the fatty acid profile of the seed oil is controlled by the genotype of the embryo (Thomas and Kondra, 1973), rather than by the maternal sporophyte genotype. This has been observed in genetic crossing experiments with plants containing contrasting levels of fatty acids as the parents. In *B.rapa* and *B. napus*, the C22:1 level is known to be under embryonic control with one and two genes, respectively (Chen and Beversdorf et al., 1990; Downey and Harvey, 1963; Harvey and Downey, 1964; Stefansson and Hougen, 1964). Complete embryonic control was reported for the C20:1 and C22:1 levels (Downey and Harvey, 1963). For the C18:1 and C18:2 levels, complete embryo control was also observed in a study although some crosses showed influence of maternal plant on these fatty acids (Thomas and Kondra, 1973).

Genetic control of the fatty acid composition completely by the embryo genotype is also reported for other crops. The C18:0 level was completely determined by the embryo genotype in reciprocal crosses between two soybean mutants and a regular cultivar. The reciprocal F_1 seeds on the two parents showed an identical level of C18:0 (Rahman et al., 1997). For some low or high C16:0 mutants of soybean, the C16:0 level is also under the control of the embryo genotype without maternal effects (Kinoshita et al., 1998; Narvel et al., 2000). In sunflower, Pérez-Vich et al. (1999) reported complete embryo control of the C18:0 level in crosses between a high C18:0 mutant and a low C18:0 line, although the influence from maternal plants was detected with crosses between the mutant line and another low C18:0 line. In safflower (*Carthamus tinctorius*), complete embryo control for the C18:1 and C18:2 levels was reported (Knowles and Hill, 1964).

2.3.2 Maternal and Cytoplasmic Effects

In addition to the control by the embryo genotype, some studies showed the influence of the maternal plant genotype and the cytoplasm on the fatty acid composition of the seed oil. The term, maternal effects, has been used to represent the two categories of effects in some reports - the effect of maternal plant genotype and the effect of cytoplasm on the fatty acid composition (Thomas and Kondra, 1973); however, it was also used to refer to only the effect of maternal plant genotype (Pérez-Vich et al., 1999; Rahman et al., 1997).

Maternal and cytoplasmic effects are detected using reciprocal crosses between parental lines with contrasting levels of a particular fatty acid (Pérez-Vich et al.,1999; Rahman, et al., 1997; Thomas and Kondra, 1973). Reciprocal crossing is conducted by cross-pollinating two parental lines with each other, thus leading to production of F_1 seeds on the two parental lines. When comparing the fatty acid levels of the F_1 seeds and the self-pollinated (SP) seeds on the same parental lines, the sporophyte genotype and cytoplasm are the same and the cytoplasm of the embryo cells is expected to be the same; only one genetic factor, the embryonic genotype of the seeds, is different. If the F_1 and SP seeds are identical in the level of the fatty acid on the same parental lines, the fatty acid is completely controlled by the maternal plant genotype (Thomas and Kondra, 1973). Conversely, when comparing the fatty acid levels of the reciprocally cross-pollinated F_1 seeds on the two parental lines, only the embryo nuclear genotype of these genetic factors is the same. Thus, if the F_1 seeds on the two parental lines produced the same level of the fatty acid, the fatty acid level is controlled completely by the embryo genotype (Downey and Harvey, 1963; Pérez-Vich et al., 1999; Rahman et al., 1997; Thomas and Kondra, 1973).

However, a significant difference in the fatty acid level between the F_1 seeds on the two parental lines could be due to different sporophyte genotypes of the two parents (maternal effect) and/or due to different cytoplasms of the embryo cells in the reciprocal F_1 seeds (cytoplasmic effect). Further comparison between F_2 seed of the reciprocal F_1 populations is required to determine whether cytoplasmic effect exists (Pérez-Vich et al.,1999; Rahman et al., 1997; Thomas and Kondra, 1973). The reciprocal F_1 populations are different in the cytoplasm of the seed embryo cells and the

sporophyte cells, whereas the nuclear genotype, on the whole, is expected to be the same (Thomas and Kondra, 1973).

Maternal and cytoplasmic effects have been observed in genetic studies where the fatty acid composition was predominantly determined by the embryo genotype as shown by significant differences between F_1 seeds and SP seeds. A study in *B. napus* (Thomas and Kondra, 1973) with three pairs of reciprocal crosses showed that, for one pair of reciprocal crosses, both the C18:1 and C18:2 levels were the same in the F_1 seeds on the two parental lines, indicating complete embryo control. For the other two pairs, the reciprocals were significantly different from each other as well as from the corresponding SP seeds, indicating maternal effects on these two fatty acids. Similar levels in C18:1, as well as in C18:2, in the F_2 seeds from the reciprocal F_1 populations excluded the influence of cytoplasm. Pleines and Friedt (1989) also observed effects of maternal plants on the C18:3 level of F_1 seeds from reciprocal crosses with a low (2.9%) C18:3 mutant from Roy and Tarr (1987) as a parent, without a cytoplasmic effect being detected. Approximately 25% of the variation was caused by the maternal effect, and the remaining was caused by the embryo nuclear genotypes. A similar estimate (20%) of the contribution from maternal effect was obtained for the variation in the C18:3 level by Rakow (1973). However, predominant control of the C18:2 level by the maternal plant genotype, with some degree of influence of the embryo genotype, was observed in another study, while the C18:1 and C18:3 levels were mainly determined by the embryo genotype (Bartokowiak-Broda and Krzymanski, 1983). Complete genetic control by the embryo genotype was reported for the C22:1 level and C22:0 level in *B. napus* (Downey and Harvey, 1963). No literature was found to show maternal effects on these two fatty acids.

In *B. napus*, a cytoplasmic effect on the fatty acid composition was observed in a genetic study with a low C18:3 mutant line (Roy and Tarr, 1987). The cytoplasmic effect influenced the level of C18:3 in both triacylglycerol (TAG) and monogalactosyl diaceylglycerol (MGDG) portions although the C18:3 level in the TAG portion was also influenced by nuclear genes. The C18:3 level in MGDG was controlled mainly by cytoplasmic inheritance (Diepenbrock and Wilson, 1987).

In sunflower, reciprocal crosses between a 25% C18:0 line produced by mutagenesis and two regular lines with 5-8% C18:0 were studied for the genetic control. One pair of the reciprocal crosses showed maternal effect and the other did not. Comparison of reciprocal F_2 seeds did not show a cytoplasmic effect (Perez-Vick et al., 1999). In flax, the C18:1, C18:2 and C18:3 levels showed maternal effect although the levels were determined largely by the embryo genotype (Yermanos and Knowles, 1962). In corn, a cytoplasmic effect was evident for the C18:1 and C18:2 levels in some crosses based on reciprocal F_1 populations (de la Roche et al., 1971; Poneleit and Bauman, 1970). In soybean, cytoplasmic effect on the fatty acid composition has also been observed (Brim et al., 1968; Jellum, 1966).

One possible cause for the effect of maternal plant on the fatty acid composition of the seed oil is the synthesis of fatty acids in the seed coat, aleurone, or endosperm layer, developed from maternal tissues. Although the fat content in these tissues is less than 6% of the total fat of the mature seed in rapeseed, under some circumstances, such as reduced seed size, the ratio could be increased (Pleines and Friedt, 1989).

Alternatively, maternal effect may be caused by physiological or agronomic characters, which directly or indirectly influence the fatty acid synthesis in the seed embryo. Translocable metabolites from maternal tissues have been proposed as possible physiological factors regulating the fatty acid synthesis in the seed (Pleines and Friedt, 1989). Influence of abscisic acid (ABA) on the C22:1 level has been reported (Holbrook et al., 1992; Wilmer et al., 1998). ABA from maternal origin, though also probably from the embryo, has been proposed to be a signal regulating C22:1 production in the seed (Wilmer et al., 1998).

Differences between parents in agronomic characters, e.g. the time of flowering, time of seed maturing and period from flowering to maturity, is another explanation for maternal effects (Thomas and Kondra, 1973). Influence of environment, especially the temperature during seed development, on the fatty acid composition, has been recorded (Wilmer et al., 1998; Craig, 1961). Phenotypic difference in the time of flowering leads to seed development under different conditions in an

environment with fluctuations in temperature.

Alternatively, maternal effects can be caused by genes which directly control fatty acid biosynthesis and are carried by the sporophyte genotype. For example, a study with soybean showed that two major genes controlled the C18:3 level of the seed oil. One gene controls the desaturation of C18:1, and the effect was governed by the genotype of maternal plants. The other gene controls the desaturation of C18:2, and the effect was controlled by the embryo genotype (Wilson and Burton, 1986).

Cytoplasmic effects on the C18:3 level in *B. napus* have been associated with maternally inherited plastome genes, as well as the contribution of the fatty acid synthesized by the plastidic mechanism (Diepenbrock and Wilson, 1987; Pleines and Friedt, 1989).

2.3.3 Environment

The fatty acid composition of *Brassica* seed oil can be significantly influenced by the environment, particularly by the temperature during seed development (Pleines and Friedt, 1988, 1989; Trèmolières et al., 1982; Wilmer et al., 1996), although other factors, e.g. soil fertility (Ahmad and Abdin, 2000) and water stress (Bouchereau et al., 1996; Pritchard et al., 2000), also have an influence.

The environmental influence on the fatty acid composition is detected when the same genotypes are grown at different locations, on different planting dates, or in contrasting environments controlled artificially. Across locations, for example, the levels of C18:1, C18:2 and C22:1 in the seed oils of several *B. napus* and *B. rapa* cultivars varied widely although the C18:3 level showed less variation (Craig, 1961). Data from advanced canola breeding experiments lasting 10 years at multiple locations in Australia also showed variation in the fatty acid composition due to different environments, with the C16:0 level being 6.4 +/- 0.1%, C18:1 being 60.3 +/- 0.4% , C18:2 being 19.7 +/- 0.3%, and C18:3 being 10.4 +/- 0.3%. Lower temperature and lower rainfall were correlated with reduced C18:1 levels (Pritchard et al., 2000).

A controlled environment study in which the photoperiod and the light intensity were the same and the temperature varied, demonstrated the effect of temperature on the fatty acid composition, the genotypic difference in the temperature sensitivity, and the different sensitivity of seeds at different developmental stages (Wilmer et al., 1996). In this experiment, the high C22:1 cultivar 'Reston' produced 30% less C22:1 when being grown continuously at 25 °C than at 15 °C after the onset of flowering, whereas the high C22:1 cultivar 'Gulle' showed much less response to the temperature. The difference of seeds at different stages in the sensitivity to temperature was detected by transferring plants from 15 to 25 °C or *vice versa*. After two thirds of the seed development had been completed, change in temperature did not influence the fatty acid composition. However, Reston plants transferred from 15 °C to 25 °C before or during the period of the maximum lipid synthesis, showed a fatty acid composition similar to that of the plants having been grown at 25 °C after the onset of flowering (Wilmer et al., 1996). Reston and Gulle plants grown at the higher temperature after flowering also showed decreased C18:3 and increased C18:1 level, with slight, but not significant, increases in the C16:0, C18:0 and C18:2 levels (Wilmer et al., 1996).

A decrease in the C22:1 level at higher or lower temperatures than the optimum temperature of 15 °C was also reported by Canvin (1965). Higher temperatures also reduce the C22:1 level in microspore-derived embryos (Wilmer, 1996).

For low C22:1 *B. napus* cultivars, in the controlled environment study by Wilmer et al. (1996), the low C22:1 cultivar 'Aurora' showed a lower C18:3 level and a higher C18:1 level at the higher temperature (25 °C), with minor changes in the levels of C18:0, C18:2 and C16:0. A significant increase in the total of C16:0 + C18:0, in addition to C18:1, was observed in environments with higher temperatures in field and controlled environments for both the conventional canola cultivar 'Regent' and the low C18:3 canola cultivar 'Stellar' (Deng and Scarth, 1998).

In soybean, the effect of temperature on the C16:0 and C18:0 levels was observed in eight field environments with contrasting mean temperatures during seed-filling (Rebetzke et al., 1996). Increases in day or night temperatures showed different effects on the fatty acid profile in a

controlled environment study. Increase of the day temperature from 30 to 35 °C resulted in higher C18:1, lower C18:2 and C18:3 levels. But the effect of the night temperature was dependent on the day temperature: a higher night temperature (30 °C) resulted in increased C18:1 and decreased C18:2 and C18:3 levels when the day temperature was controlled at 35 °C. In contrast, when the day temperature was set at 30 °C, the higher night temperature (30 °C) led to decreased C18:1 and increased C18:2 level (Gibson and Mullen, 1996). Higher C18:1 and lower C18:3 levels at higher temperatures during the seed-fill stage were also reported for plants grown on a wide range of planting dates (Kane et al., 1997). In sunflower, the fatty acid composition of both high oleic acid cultivars and high linoleic acid cultivars is sensitive to crop management, and soil and climate variations (Lagravere et al.,1998).

The effect of temperature on the levels of unsaturated fatty acids has been associated with the activity of fatty acid desaturases, which is probably regulated by change in the solubility of oxygen and the fluidity of the membrane, or by differential expression of the desaturase genes, at different temperatures (Browse and Slack, 1983; Trèmolières et al., 1982). However, the activity of the elongase, the enzyme synthesizing C22:1, is poorly correlated with the level of C22:1 in mature microspore-derived embryos (Wilmer et al., 1998). In addition, temperature may influence fatty acid levels of the seed oil through the maternal plant. Plants at higher temperatures (24.5/18 °C, day/night) showed a higher degree of maternal effect on the C18:3 level than those under a cold condition (14.5/8 °C) in a study with *B. napus* (Pleines and Friedt, 1989).

2.4 *Agrobacterium*-Mediated Genetic Transformation

Agrobacterium-mediated T(transferred)-DNA transfer is the first and the most widely used method for the introduction of foreign genes into dicotyledonous plants (Dale and Irwin, 1994; Day and Lichtenstein, 1992; Walden and Wingender, 1995), although alternative methods are available, e.g. microprojectile bombardment, direct DNA uptake by protoplasts, microinjection, electrophoresis, silicon carbide fiber, electroporation of intact tissue, and plant viral genome-based methods (Birch, 1997; Hinchee et al., 1994; Koffr et al., 1998; Songstad et al., 1995; Tomes et al., 1995). Compared to other methods, *Agrobacterium* transfer systems do not rely on protoplasts and special delivery

equipment, and the occurrence of complex integration patterns in the genome of transformed plants is relatively less frequent (Birch, 1997; Day and Lichtenstein, 1992; Walden and Wingender, 1995).

2.4.1 *Agrobacterium tumefaciens*

A. tumefaciens is the causative agent of crown-gall tumor diseases in wounded gymnosperm and dicotyledonous angiosperms (dicots) (Day and Lichtenstein, 1992; Zupan and Zambryski, 1995). Oncogenic strains of *A. tumefaciens* contain a Ti (tumor-inducing) plasmid of 150-250 kb, first identified in 1974 (Chilton, 2001). The Ti plasmid has a T-DNA region defined by two 25-bp directly repeated sequences - the left border and right border. T-DNA possesses genes for the syntheses of auxin, cytokinin and opine. Oncogenic genes can be transferred into plant cells and stable integrated into the genome. The expression of the transformed *onc* genes causes the disease (Day and Lichtenstein, 1992; Zupan and Zambryski, 1995).

2.4.2 T-DNA and the Genes Involved in Transformation

The introduction of T-DNA into the plant genome is due to the combined actions of the T-DNA borders, gene products encoded by virulence (*vir*) genes located outside the T-DNA region on the Ti plasmid, together with certain genes in the bacterium and plant genomes (Gelvin 2000). The right and left borders of T-DNA act as the initiation and termination points of transfer, respectively (Day and Lichtenstein, 1992; Gelvin, 2000; Zupan and Zambryski, 1995). The *vir* genes, with regard to their functions in the transformation process, can be located in the same plasmid with the T-DNA as in *in cis* vector systems, or in another disarmed Ti plasmid in which the T-DNA region and the borders have been deleted as in the commonly used binary vector systems (Dale and Irwin, 1994; Day and Lichtenstein, 1992; Firoozabady and Kuehnle, 1995). The transformation of T-DNA is not affected by the deletion of the T-DNA genes although plant growth factors, as encoded by the T-DNA genes, are required for the proliferation of transformed cells (Day and Lichtenstein, 1992; Joos et al., 1983; Zambryski et al., 1989). Therefore, oncogenic genes in the T-DNA region can be deleted, and desired genes can be inserted between the two borders for transformation (Dale and Irwin, 1994). Growth factors are provided in vitro when artificial T-DNA is used for transformation (Zambryski et al., 1989).

2.4.3 Transfer of T-DNA to the Plant Cell

As the initiation step of the transformation process, *Agrobacteria* perceive a signal *via* the VirA sensory protein, possibly together with some proteins encoded by the *Agrobacterium* chromosome. The signal is likely phenolic compounds and sugar compounds released by wounded cells, (Day and Lichtenstein, 1992; Gelvin, 2000). The VirA protein is membrane bound and can transmit the extracellular signal to the intracellular *vir*G gene product, which in turn acts as the inducer for the rest of the *vir* genes (Day and Lichtenstein, 1992; Gelvin, 2000). Most of the induced Vir proteins are directly involved in the T-DNA transfer process, such as proteins VirC, D, E, J, F (Gelvin, 2000). VirC1 is the putative binding protein of the 'overdrive' sequence that is adjacent to the right border but outside the T-DNA region, and the binding may enhance the efficiency of transfer (Day and Lichtenstein, 1992; Gelvin 2000; Ooms, 1992). VirD1 and VirD2 together produce a single-stranded endonucleolytic cleavage in the lower strand of the T-DNA at the two borders (Day and Lichtenstein, 1992; Zupan and Zambryski, 1995). These nicks are used as the initiation and termination sites for T-strand transfer (Zupan and Zambryski, 1995). After nicking, VirD2 strongly associates with the 5' end of the T-strand, and guides the T-strand from the bacterium into the plant cell (Gelvin, 2000). VirE2 was originally proposed to be part of the "T-complex" that is also composed of the T-strand and the VirD2 protein. Recent studies suggest that VirE2 may function only in the plant cell because the T-strand/VirD2 can be transferred separately from VirE2 (Gelvin, 2000). Both VirD2 andVirE2 contain plant-active localization signal sequences, which may, together with some plant proteins, determine the nuclear localization of T-DNA (Gelvin, 2000).

2.4.4 Integration

Little is known about the precise mechanism of T-DNA integration into the plant genome (Gelvin, 2000), but it is widely believed that T-DNA integration occurs in random positions by illegitimate recombination (Gelvin, 2000; Koncz et al., 1994; Zupan and Zambryski, 1995). This is the major mode for foreign DNA integration into plant and mammalian cells (Gelvin, 2000; Ohl et al., 1994), which does not require significant sequence homology between the transforming and the targeted DNA (Ohl et al., 1994). Illegitimate recombination occurs approximately 100,000-fold more frequently than does homologous recombination (Gelvin, 2000). In the random integration process,

VirD2 and VirE2 may also play a role. It is not yet clear whether T-DNA integrates via strand invasion of locally denatured plant DNA by single-stranded T-strand, followed by second strand repair synthesis, or whether the T-strand becomes double stranded before integration into plant genome (Gelvin, 2000).

2.4.5 Plant Regeneration

Regeneration of transformed cells, preferentially over non-transformed cells, is achieved by the inclusion of a selectable marker gene in the T-DNA region, so that transformed cells expressing the marker gene are resistant to an antibiotic and can be regenerated on medium supplemented with the antibiotic. The most commonly used selectable marker is neomycin phosphotransferase gene (npt II) from transposon Tn5 (Bevan et al., 1983), which confers resistance towards some aminoglycosides, such as kanamycin, G418 (a kanamycin analog) (Knutzon et al., 1992), neomycin, and gentamycin (Poulsen, 1996; Dale and Irwin, 1994). The content of kanamycin to be used depends on the source of explants. Winter type *B. napus* cultivars, for instance, tend to be more resistant to kanamycin than spring type. Lower levels of antibiotics allows more untransformed cells to 'escape' and regenerate (Poulsen, 1996). In a canola transformation experiment with an *E.coli* KAS II gene and npt II gene, 13% of the regenerated plants did not carry the transgenes, as determined by PCR and Southern blotting analyses (Verwoert et al., 1995). For *B. napus* transformation, the concentration of kanamycin in the selection media could be 15 to 100 mg/l (Hinchee et al., 1994). In some experiments, 25 mg/l kanamycin was added to the callus induction and shoot regeneration media, with 50mg/l in media for root induction and subsequent regeneration (Knutzon et al., 1992).

2.4.6 Integration Pattern

There is currently no control over the integration process of T-DNA into a plant genome (Gelvin, 2000). Analysis of transformed plants showed that the target sites of T-DNA are preferentially located in transcriptionally active regions of the plant genome; the chromatin conformation in these regions may be more 'open' for T-DNA integration (Gelvin, 2000). More than 90% of T-DNA insertions could occur in transcriptional units (Birch,1997), although the percentage varies from

experiment to experiment (Bhattacharyya et al., 1994).

Transformed plants may contain one or multiple inserts in the genome. The insert can be an intact T-DNA copy or an incomplete copy truncated at the ends. Vector backbone sequences can be linked or unlinked to T-DNA. Multiple copies can be at different segregating loci or in the same genomic site (Birch, 1997; Hinchee et al., 1994; Koncz et al., 1994; Matzke et al., 1994). Complex integration patterns, distinct from single-copy insertion of an intact T-DNA copy, are often detected in transformed plants (Birch, 1997; Hinchee et al., 1994; Matzke et al., 1994; Ooms, 1992)

Integration of multiple copies into the same genomic site occur frequently (De Neve et al., 1997; Maqbool and Christou et al., 1999), and the copies are normally present in a head to tail arrangement with differing unit lengths (Ooms, 1992). But inverted repeats, either head to head or tail to tail, can be present (Bauer et al., 1998; Day and Lichtenstein, 1992; Fladung, 1999; Muskens et al., 2000; Stam, 2000).

Usually, inserted T-DNA conservatively maintains the sequences between the two borders although both the ends may suffer small deletions (Koncz et al., 1994). However, integration of incomplete T-DNA copies truncated at their ends is not unusual (Dominguez et al., 2000; Koncz et al., 1994; McCabe et al., 1999). Integration of incomplete T-DNA copies has been associated with presence of a 'pseudoborder' within the T-DNA region (Day and Lichtenstein, 1992). Thus, transgenic plants expressing the marker gene do not necessarily carry the transgene of interest (Hinchee et al., 1994).

Integration of vector backbone sequences has been detected in some studies (Jakowitsch et al., 1999; Kononov et al., 1997; Martineau et al., 1994). The backbone sequence inserted into the plant genome can be linked to, or independent of, the two borders of the transformed T-DNA (Kononov et al., 1997). Thus, it has been proposed that VirD2 protein can bind to the 5' ends of the lower and the upper strands of T-DNA, as well as to the non-T-DNA strands, then ViD2 guides the transfer to the plant cell (Kononov et al., 1997). This view is contrary to the widely known transformation process in that only the lower strand of T-DNA is transferred and proceeded from the right to the left

border (Gelvin 2000; Zupan and Zambryski, 1995). The frequency of integrations containing backbone sequences is surprisingly high in some experiments (Dale et al., 1998). In a *Petunia* transformation experiment, 15% of transformants contained backbone sequences (Cluster et al., 1996). In tobacco, a frequency of 75% was reported (Kononov et al., 1997). In *B. napus*, 15 out of 31 transformed plants carried up to 355 bp of the vector sequences (Dale et al., 1998).

The frequency for the occurrence of complex integration pattens can be very high. Approximately 30-50% of the inserts are estimated to be single copies, and the remaining transformants can contain multiple inserts present at one locus (often in inverse orientation or complex arrangements) or at multiple segregating loci (Matzke et al., 1994). In another experiment, 80% of the transformed plants had more than one copy, and 30% of the inserts were incomplete or rearranged (Day and Lichtenstein, 1992). These frequencies are influenced by the strain of *Agrobacterium*, the vector system, and the source of explants (Day and Lichtenstein, 1992; De Neve et al., 1997; Grevelding et al., 1993).

In addition, the plant genomic DNA flanking the integrated T-DNA may also suffer deletion, rearrangement, perfect or imperfect, direct or indirect repeat (Hinchee et al., 1994; Koli et al., 1998; Koncz et al., 1994; Takano et al., 1998).

2.5 Modification of the Fatty Acid Composition by Genetic Transformation

The developments in genetic transformation and related technologies, the clarification of plant fatty acid biosynthetic pathways, and the significant alteration in the fatty acid composition of the seed oil which crop plants can tolerate with little or no negative effect on the agronomic performance (Knutzon et al., 1992; Murphy, 1994), have facilitated manipulation of the fatty acid composition of oilseed crops by genetic engineering approaches (Murphy, 1994; Poulsen,1996; Scarth and McVetty, 1999; Somerville, 1993, Töpfer et al., 1995). Significant modifications of the fatty acid composition in *B. napus* have been achieved by introducing foreign genes into existing cultivars via *Agrobacterium*-mediated transformation. The genes transformed include those coding for the enzymes which play important roles in the fatty acid biosynthesis or in the formation of

triacylglycerols (TAG), e.g. acyl-ACP thioesterase, desaturase, elongase, acyl-transferase, KAS, and ACCase. Transgenic lines producing modified oils with enhanced or decreased levels of some fatty acid(s), or with novel fatty acids which formerly accumulate only in the seed oil of wild plant species, e.g. medium-chain fatty acids (MCFA), have been developed (Alonso and Maroto, 2000; Gunstone, 1999; Töpfer et al., 1995).

2.5.1 Medium-Chain Fatty Acids

Plants oils with enriched MCFA are useful in a number of food and non-food industries (Martini et al., 1995; Ohlrogge, 1994; Töpfer et al., 1995). Dietary fats, which are an important energy source for infants, are manufactured from C8:0 and C10:0 in an expensive industrial process that forms medium-chain TAGs (Töpfer et al., 1995). C12:0 has an ideal surfactant property because this chain length provides a balance of solubility in both aqueous and non-aqueous environments, and is widely used as a raw material in the production of soap, detergent, and shampoo (Ohlrogge, 1994; Töpfer et al., 1995). In addition, oils rich in C12:0 can be used in manufacturing of food products such as fillings, margarines, spreads, shortenings, and as commercial frying oils (Health Canada, 1999).

The commercial sources of MCFA in the past were mainly coconut and palm kernel oils (Brosten, 1996; Ohlrogge, 1994;Töpfer et al., 1995). Major oilseed crops accumulate predominantly long-chain fatty acids with a length of 16 and 18 carbon atoms in the seed oil (McVetty and Scarth, 2002; Töpfer et al., 1995). In canola oil, MCFA content is less than 0.2% (Voelker et al., 1996).

Certain genera or species of angiosperm families produce oils containing more than 50% MCFA in the total fatty acids (Hilditch and Williams, 1964; van de Loo et al., 1992). *Ulmaceae* accumulates C8:0 and C10:0; many *Lauraceae* species produce C10:0 and C12:0; *Myristicaceae* often deposits C14:0. Palms (*Aracease*) and the genus *Cuphea* (*Lythraceae*, Graham et al., 1981) contain species displaying the whole range of MCFA (Graham, 1989; Graham et al., 1981; Voelker et al., 1997). In *Cuphea*, some species have 93% MCFA in the seed oil (Graham, 1989). Mexican shrub (*C. hookeriana*) accumulates up to 60% caprylic acid (C8:0) and 25% capric acid (C10:0) (Dehesh et al., 1996; Graham et al., 1981), whereas *C. lanceolata* produces up to 83% C10:0 and *C. wrightii*

accumulates 29% C10:0 and 54% C12:0 (Hilditch and Williams, 1964; van de Loo et al., 1992). Although domestication of these plant species as crops may provide a new source for MCFA, an alternative approach is to genetically engineer an established oil crop, such as rapeseed, to produce them (Martini et al., 1995).

The accumulation of MCFA in seed oils has been associated with the function of specialized TE (Hawkins and Kridl, 1998; Jones et al., 1995; Ohlrogge et al., 1978; Pollard et al., 1991). At least one TE gene which codes for a TE with a substrate specificity closely corresponding the MCFA composition has been cloned from a number of plant species (Voelker et al., 1997). As demonstrated in transgenic plants, these TE are, at least in part, responsible for the characteristic fatty acid compositions of their respective species (Hawkins and Kridl, 1998).

Lauric Acid: This MCFA was produced by plants which do not naturally accumulate such a component in the seed oil by introducing a TE gene into *Arabidopsis* plants (*A. thaliana*) and *B. napus* plants (Voelker et al., 1992, 1996). The TE gene, later named *UcFatB1* (GenBank accession No. M94159 (Jones et al., 1995), was cloned from California bay tree (*U. californica*) (Voelker et al., 1992), a species which can accumulate up to 70% C10:0 and C12:0 in the seed oil (Pollard et al., 1991).

For seed-specific expression, the bay-TE cDNA was inserted into an expression cassette containing the seed-specific napin promoter and termination sequence in the plasmid pCGN3223 (Kridl et al., 1991). The napin promoter and terminator were originally from *B. rapa*. The chimeric gene was engineered into the T-DNA region in a binary transformation vector pCGN1578, which has the selectable marker npt II gene on the left side (McBridge and Summerfelt, 1990). The introduction into plants was fulfilled via the *A. tumaficiens*-mediated transformation method (Radke et al., 1988). Transformed *A. thaliana* plants could accumulate C12:0 and C14:0, accompanied by reductions in most of the other fatty acids including C18:0, C18:1, C18:2 and C20:1 in the seed oil; but the total seed fatty acid amount by weight was not significantly changed (Voelker et al., 1992).

In transformed low-erucic acid *B. napus* cv. 212/86 plants, the expression of the bay-TE under the same expression cassette as describe above was detectable in mid-maturity seeds at a level of 0.015% of the total protein (Voelker et al., 1996; Health Canada, 1999). In more than 100 primary transformants, the C12:0 level ranged from the background level (0.02% C12:0) to 40%, with most plants having 5-20% C12:0; also, C14:0 was increased from background (0.15%) to 5% (Voelker et al., 1996). In the next generation produced by self-pollination, some plants with up to 56% C12:0 in the seed oil were identified. The increase in C12:0 was accompanied by a proportional increase of C14:0, indicating the C14:0-ACP pool was not reduced sufficiently. With the increase in the C12:0 level, there were corresponding declines in the levels of C16:0, C18:0, C18:1 and C18:2; but the relative proportions of these fatty acids changed only slightly, indicating that, when fatty acid elongation was terminated at C12:0, the fatty acid biosynthetic pathway through to unsaturated C18 fatty acids operated quantitatively as in the control plants. Similar to the expression in *A. thaliana*, the C18:3 level of transformed *B. napus* plants was only slightly changed. The maintenance of the C18:3 level was also observed with the expression of a C16:0-ACP TE in *B. napus* (Jones et al., 1995).

The seed oil of the bay-TE transformed *B. napus* plants resembles coconut and palm kernel oils in the level of MCFA (Voelker et al., 1996). About 2,000 acres of bay-TE transgenic canola was grown for the first time in Southern Georgia, U.S. in 1995 (Brosten, 1996; Töpfer et al., 1995). In 1999, about 90,000 acres were produced in North Dakota and Georgia, U.S. (Johnson, 1998). The two lines released for field experiments in Canada originated from a primary transformant carrying at least 15 copies of the bay-TE transgene at five independent loci, and they can accumulate up to 40% C12:0 and 4% C14:0. Tests of three generations showed stable inheritance of the bay-TE transgene since there was no change in the banding pattern on Southern blots (Canadian Food Inspection Agency, 1998). The Canadian Food Inspection Agency (1998) assessed the environmental safety and livestock feed safety, and it approved undefined release of 'laurate canola' and the meal product for use as canola meal. Health Canada (1999) assessed the environmental safety and had no objection of the refined oil for human consumption.

Other Medium-Chain Fatty Acids: Five different TE genes were cloned from *C. lanceolata* (Martini et al., 1995). Transformation of *B. napus* with one of the TE genes under the control of its own promoter led to accumulation of 1% C8:0 and 3% C10:0 in the seed oil, with slight decreases in the C18:1 level and C18:2 level. Transformation with another gene produced plants with 7% C14:0, accompanied by 15% C16:0, a decrease in C18:1 and an increase in C18:2 (Martini et al., 1995). No obvious changes were noted in the C18:0 and C18:3 levels.

Transformation of *B. napus* plants with the TE gene *Ch FatB2*, cloned from *C. hookeriana*, led to accumulation of 4-7% C8:0 and 12-16% C10:0 with the highest expression of 11% C8:0, 27% C10:0 and 2% C12:0 in the seed oil (Dehesh et al., 1996). *Cinnamomum camphorum* belongs to the same family (*Lauraceae*) as California bay trees. It accumulate predominantly C10:0 and C12:0 in the seed oil (Yuan et al., 1995). Canola plants expressing the *Cc FatB1* TE gene cloned from *C. camphorum* could accumulate 22% C14:0 in the seed oil (D.J.H., Kridl, and T.A.V., unpublished, based on Yuan et al., 1995). A different TE isoform could be responsible for the high C10:0 and C12:0 levels in *C. camphorum* seed oil (Yuan et al., 1995).

2.5.2 Palmitic Acid

Palmitoyl (C16:0)-ACP is at a branch point in the fatty acid biosynthetic pathway. It can be elongated by KAS II, or released by a TE and then enter the storage oil. The flux of C16:0 is controlled in large part by the relative activities of the elongation and the TE reactions in most oilseed species (Ohlrogge, 1994). Either increase or decrease in the C16:0 level of seed oils has been achieved by transformation with TE or KAS II genes.

The cuphea-TE gene *Ch FatB1* (GenBank Accession No. U17076) codes for a 416-amino acid polypeptide, which prefers C16:0-ACP, with only minor activities on C14:0, C18:0 and C18:1-ACP (Jones et al., 1995). The expression of *Ch FatB1* under the control of the napin expression cassette of pCGN3223 (Kridl et al., 1991) in *B. napus* cv. 212/86 plants led to seed oils enriched in C16:0. Among 25 independent transformants, C16:0 levels ranged from 7 to 34%, C14:0 from 0.14 to 1%, with slight increases in C18:0 and C20:0, whereas the control plants (*B. napus* cv. 212/86) had only

traces of C12:0(0.02%) and C14:0 (0.14%), and 6% C16:0. The variation from transformed plant to plant has been associated with the influence of transgene copy number and genomic position (Jones et al., 1995).

Elm (*Ulmus americana*) seeds contain an oil with 65% C10:0 and 10% C8:0 (Davies, 1993). A cDNA library of maturing elm seeds, harvested from the boulevard trees in Davis, California, was screened with a PCR fragment as the probe. The PCR fragment was obtained by PCR reaction with elm-seed cDNA as the template and primers designed based on the conserved peptide sequences of several already sequenced TE genes (Jones et al., 1995; Voelker et al., 1997). A cDNA (*UaFatB1*, Genbank accession no. U65644) was cloned and sequenced. The derived peptide sequence showed the signal for plastidial targeting missing (Voelker et al., 1997). For expression of the TE gene *Ua FatB1* in *B. napus*, a 240-bp fragment at the 5' end was digested away and replaced by a fragment of *Ch FatB1* coding for a full-length transit peptide (Voelker et al., 1997). The resulting sequence of *Ua FatB1* attached with the *Ch FatB1* targeting sequence was inserted into the napin expression cassette and was transformed into *B. napus* cv. 212/86 by a pCGN1578-based binary vector system (Voelker et al., 1997). Transformed plants showed increases in the levels of most saturates, especially C16:0, which ranged from 15-33%. As the C16:0 level increased, C18:0 increased from about 2 to 4%, C14:0 from near 0 to 13%, C12:0 to 1.5%, C10:0 to 4%, with traces of C8:0 and a slight decline in the C18:2 and C18:3 levels (Voelker et al., 1997).

An increased level of C16:0 in the seed oil of canola plants was also achieved by transformation with a TE genes cloned from nutmeg plants (*Myristica fragranceae*) (Voelker et al., 1997), which is a species accumulating 80% C14:0 in the seed oil (Hilditch and Williams, 1964). The TE gene (*Mf FatB1*, GenBank accession no. U65642) was cloned from a cDNA library of maturing seeds in developing nutmeg fruits collected from Hawaii by screening the library with a fragment of the *UcFatB1* TE gene as the probe. Transformation of *B. napus* cv. QO4 with *Mf FatB1* followed the same strategy as described for the transformations of the bay-TE *Uc FatB1* (Voelker et al., 1992), cuphea-TE *Ch FatB1* (Jones et al., 1995) and elm-TE *Uc FatB1* TE gene (Voelker et al., 1997), with the same napin expression cassette and pCGN1578-based binary vector. The expression of the

nutmeg-TE gene increased the C16:0 level from 4.5% in the control plants to 33%, C14:0 from 0.1 to 20%. Most transgenic plants produced 8-20% C14:0, and the levels of all saturated fatty acids from C12:0 and C22:0 were positively correlated with the C14:0 level (Voelker, et al., 1997).

Although plant oils with high levels of saturated fatty acids have advantages in manufacturing of some food products, e.g. margarine and shortening (Hawkins and Kridl, 1998; Kinney, 1996), reducing saturated fatty acids in food is a dietary goal (Ohlrogge, 1994). Expression of a KAS II gene in *B. napus* resulted in a reduction in the C16:0 level (Bleibaum et al., 1993). Antisense suppression of a TE gene in soybean plants reduced the TE activity and resulted in a 2-fold decrease in the level of saturated fatty acids (Yadav et al., 1993). Expression of a rat or a yeast C18:0-CoA desaturase gene in tobacco plants slightly reduced the saturated fatty acid level but the C16:1 level was increased significantly (Grayburn et al., 1992; Polashock et al., 1992).

2.5.3 Stearic Acid

Several plant species have been identified that accumulate a significant amount of stearic acid (C18:0) in their seed oil. Such oils are valuable for shortening, margarine, and confectionery applications - as cocoa butter or cocoa butter substitutes (Hawkins and Kridl, 1998). These species include the tropical fruit tree species highly valued in south-east Asia, mangosteen (*Garcinia mangostana*), which can accumulate up to 56% C18:0 in its seed oil by weight, in addition to mango (*Mangifera indica*), cocoa (*Heobroma cacao*), shea (*Butyrospermum parkii*), sal (*Shorea robusta*), and kokoum (*Garcinia indica*). Canola cultivars have only 1.1-2.5% C18:0 in the seed oil (Hawkins and Kridl, 1998).

Transgenic canola plants accumulating higher levels of C18:0 in the seed oil have been obtained using several approaches, including transformation of FatA TE genes cloned from high-C18:0 producing species or from oilseed crop plants (Hitz et al., 1995; Hawkins and Kridl, 1998), transformation of FatB TE genes cloned from MCFA producing species (Voelker et al., 1997), sense or antisense suppression of Δ9-desaturase (Fader et al., 1995; Hitz et al., 1995), and a combination of TE expression and Δ9-desaturase suppression (Töpfer et al., 1995).

A FatA TE gene, *Garm FatA1*, cloned from a mangosteen seed cDNA library, encodes a TE with a much higher activity on C18:0-ACP than on 16:0-ACP, while preferring C18:1-ACP. Its expression in *B. napus* cv. Quantum resulted in seed oils with C18:0 levels ranging from background to 22% in 80 primary transgenic plants. The increase in C18:0 was primarily at the expense of C18:1 with a slight decline in C18:3 and C18:2, a slight increase in C20:0 and C22:0; but C16:0 and the oil content of the seed were unchanged (Hawkins and Kridl, 1998). The increase in the C18:0 level was positively correlated with the TE activity in developing seeds. Expression of a mutant of *Garm FatA1*, produced by site-specific mutation, led to accumulation of 55-68% more C18:0 than did the expression of the wild-type enzyme in transgenic *B. napus* plants (Facciotti et al., 1999). However, no TE either similar to the mangosteen *Garm* FatA TE or specific to C18:0-ACP was identified from mango - another C18:0 producing species(Hawkins and Kridl, 1998; Voelker et al., 1996). Thus, a special FatA TE isoform, as identified in mangosteen, may not be the sole determinant or not a widely adopted mechanism for C18:0 producing species. Altered activities of a Δ9-desaturase and/or TE and /or KAS II could be the contributor to the high C18:0 level in some high C18:0 species (Hawkins and Kridl, 1998).

Transformation of FatA TE genes cloned from oilseed crop plants showed distinct results compared to the result obtained with mangosteen *Garm FatA1* although in some cases the C18:0 level in the transgenic plants was increased. In soybean, over-expression of a canola FatA TE gene increased C18:0 by 3.3-fold, but also increased C16:0 by 1.5-fold, at the expense of C18:1 (T. Kinney, unpublished, based on Hawkins and Kridl, 1998). In *B. napus*, expression of a soybean C18:1-ACP TE in 'Westar' increased the C18:0 level from 1.8% of the control to 10.1% and C16:0 from 3.9% to 9.2% (Hitz et al., 1995). The expression of safflower FatA1 in *Brassica* led to a 2-fold increase of C16:0 but with no change in C18:0 (Kridl and Thompson, unpublished, based on Hawkins and Kridl, 1998). These results indicate that FatA TE of different sources are different in substrate specificity although all prefer C18:1-ACP (Hawkins and Kridl, 1998; Jones et al., 1995).

Expression of FatB TE genes in transgenic *B. napus* plants mainly increase MCFA and C16:0, but increased accumulation of C18:0 was observed in seeds of transgenic plants expressing either the

elm-TE or the nutmeg-TE transgene (Voelker et al., 1997).

Δ9-desaturase converts C18:0-ACP to C18:1-ACP in the plastid (Knutzon et al., 1992). Suppression of the activity of Δ9-desaturase in order to reduce the competition for the substrate from the desaturation pathway was achieved using one of the two most common strategies: antisense and sense suppression (Fader et al., 1995). For antisense suppression, a cDNA coding for *B. rapa* Δ9-desaturase was inserted in antisense into two expression cassettes: one with the regulatory sequences from a *B. rapa* napin gene and the other from a *B. rapa* ACP gene (Knutzon et al., 1992). The two chimeric genes were then inserted in tandem into the T-DNA region for transformation. Expression in *B. rapa* and *B. napus* plants reduced the Δ9-desaturase activity. In most transgenic *B. rapa* plants pollinated with wild-type plants, based on individual seeds, the highest C18:0 level ranged from 3 to 9%, with the highest up to 32% in some seeds. The seeds from the best plant were divided into two distinct classes, some with increased levels (21.5-32%) of C18:0 and the others with C18:0 levels equivalent to the control seeds (1.0-1.6%). The increased C18:0 level in the high C18:0 seeds was accompanied by a slight increase in long-chain fatty acids (C20:0 and C22:0) from near zero to 2-5%, with a significant decrease in C18:1 from approximately 60 to 15%, a slight decrease in C18:2 and an unchanged level of C16:0. In *B. napus*, most primary transgenic plants produced 3-10% C18:0 in the seed oil. Self-pollinated seeds showed a continuous variation in the C18:0 level ranging from 2 to 40%. Similarly, the increased 18:0 level was accompanied by a decrease in C18:1, and increases in C18:3 and the long-chain saturated fatty acids.

Sense-suppression of Δ9-desaturase activity by transformation with a soybean Δ9-desaturase gene increased the C18:0 level in *B. napus* cv. Westar from 1.8 to 12.5%, with decreases in the C18:1, 18:2 and C18:3 levels and a slight increase in C22:0 (Hitz et al., 1995). From the same transformation experiment, however, some plants showed over-expression, rather than suppression, of the activity of Δ9-desaturase. The C18:0 level of over-expressed plants was reduced to 1.1% from1.8% of the control plants, with an increase in the C18:1 level (Hitz, et al., 1995).

Incorporation of the two approaches, over-expression of the TE activity and suppression of the Δ9-

desaturase activity, showed combined effects on the C18:0 level in transgenic soybean plants. Over-expression of a long chain-specific TE gene increased the C18:0 level to 11%. Crossing this line with a second transgenic line containing 13 % 18:0 due to down-regulation of the Δ9-desaturase activity, resulted in a seed oil with 45% C18:0 (Broglie and Hitz, unpublished, based on Töpfer et al., 1995). The results clearly indicate that the long-chain TE and Δ9-desaturase compete for the common substrate, C18:0-ACP (Töpfer et al., 1995).

2.5.4 Oleic Acid

Suppression of Δ12-desaturase could increase the level of C18:1 in the seed oil of *B. napus* and soybean. In *B. napus*, the homologous Δ12-desaturase gene was transformed into *B. napus* cv. Westar (Hitz et al., 1995). Transgenic plants which co-suppressed the Δ12-desaturase gene accumulated C18:1 up to 84.1%, with a decline in C18:2 to 5.2%, C18:3 to 2.9%, and no obvious change in the other fatty acids, in comparison to 67% C18:1, 19.0% C18:2 and 7.5% C18:3 in the control plants. However, plants over-expressing Δ12-desaturase, originated from the same transformation experiment, produced less C18:1 (33.3%) and more C18:2 (49.2%) than the control, with no obvious change in the other fatty acids (Hitz et al., 1995). Antisense repression of Δ12-desaturase displayed a result similar to the co-suppression, with the C18:1 being increased up to 83% (Hitz et al., 1995). This line was crossed with a mutant line 'IMC29' accumulating 78% C18:1, resulting in the development of a *B. napus* line with 88% C18:1 (Hitz et al., 1995; Töpfer et al., 1995).

In soybean, transgenic lines carrying an antisense Δ12-desaturase gene were developed by introducing the homologous gene in an antisense orientation using the gene gun technology. The gene gun delivers microprojectiles coated with the DNA coding for the promoter and the gene of interest into plant cells. Expression of the antisense gene increased the C18:1 level to 76.1%, decreased C18:2 to 2.5%, compared to 21% and 55% in the seed oil of the control plants, respectively. The C16:0 level was decreased slightly, and the C18:0 and C18:3 levels were almost unchanged (Fader et al., 1995).

2.5.5 Erucic Acid

High erucic acid rapeseed (HEAR) cultivars have less than 1% C22:1 being incorporated into the central position (*sn*-2) of the glycerol backbone due to the poor affinity of rapeseed acyltransferase LPAAT to very long chain fatty acids, including C22:1 (Brough et al., 1995; Lühs et al., 1999). However, it is known that meadowfoam (*Limnanthes douglasii*) seeds accumulates trierucin, TAGs with C22:1 in all the three positions of the glycerol molecule, due to a specific LPAAT (Cao et al., 1990; Lohden and Frentzen, 1992; Murphy, 1994; Töpfer et al., 1995). Expression of the meadowfoam LPAAT in high erucic acid *B. napus* plants resulted in trierucin accumulation (Lassner et al., 1995; Lühs et al., 1999; Weier et al., 1997); but it did not increase the total C22:1 level in HEAR oil. This could be due to the acyl-CoA pool in the seed of HEAR plants being too low to support high levels of tirerucin synthesis (Lühs et al., 1999). Manipulation of genes in the elongase complex and in the pathway before elongation of C18:1 or desaturation has been proposed for development of rapeseed oil with very high C22:1 levels (Lühs et al., 1999; Töpfer et al., 1995).

2.6 Inheritance and Expression of Transgenes

2.6.1 Inheritance of Transgenes

It is important that transgenes are inherited and expressed in a predictable, consistent, and stable manner for practical applications (Conner and Christey, 1994; Conner et al., 1998). Numerous studies have shown that transgenes can be stably transmitted for generations through meiosis, and segregate in Mendelian fashion (Day and Lichtenstein, 1992; Scott et al., 1998; Webb et al., 1999). Even silent transgenes can be stably inherited (McCabe et al., 1999). Unstable, non-Mendelian transmission of transgenes, however, has been observed. In most cases analyzed at the molecular level, non-Mendelian ratio of the phenotypic classes was caused by transgene inactivation (Finnegan and McElroy, 1994); but in some cases, segregation distortion cannot be explained by transgene inactivation. The possible causes include: integration of transgenes into essential plant genes; linkage with deleterious or lethal alleles (Scott et al., 1998); loss of the transgene due to rare, unusual meiotic events (Conner et al., 1998; Scott et al., 1998); somaclonal variation (Bean et al., 1997; Joersbo et al., 1999); and homologous recombination (Conner et al., 1998; Lichtenstein, 1994).

Non-Mendelian segregation is not specific to transgenes. Some plant endogenous genes also segregate in non-Mendelian manners (Bradshaw and Stettler, 1994; Konish et al., 1992; Scott et al., 1998). The frequency of mitotic and meiotic losses, tested with single-locus homozygous transgenic lines in several plant species, is well below the accepted limit for off-types permitted in seed production of self-pollinated cultivars (Conner et al., 1998). Intrachromosomal recombination (ICR) has been documented in several reports where transgenic plants carried direct repeats or inverted repeats (Conner et al., 1998). However, the frequency of ICR in plants is very low, usually ranging from 10^{-4} to 10^{-7} (Conner et al., 1998; Lichtenstein, 1994).

2.6.2 Expression of Transgenes

The terms 'stability' and 'instability' have been used in literature to describe variability in the expression of transgenes from plant to plant, from cell to cell, change in expression states between activation and inactivation, as well as variation due to environmental factors (Baulcombe, 1996; Cannell et al., 1999; Charrier et al., 2000; Dale, 1998; De Neve, 1999). High, stable expression of transgenes has been observed in many studies. Transgenic canola lines with multiple copies (5 to15) of the bay-TE gene were maintained stable for more than five generations without any apparent genetic instability and loss of expression (Voelkere et al., 1996). Stability in the expression across generations has also been reported for other plant species with various transgenes (Fearing et al., 1997; McCabe et al., 1999; Scott et al., 1998). The expression level of a transgenic CryIA(b) gene, for example, in a maize inbred and two hybrid lines, were stable over successive backcross generations, without obvious difference among BC_1, BC_2, BC_3 and BC_4 populations planted concurrently (Fearing et al., 1997).

However, there are examples of unstable expression and wide variation in the expression level of transgenes. Primary transformants from independent transformation events commonly show wide variation in the expression level (Day and Lichtenstein, 1992; Bhattacharyya et al., 1994; Hobbs et al., 1990; Maqbool and Christou, 1999). Independent transformants may carry a simple single T-DNA copy or multiple copies. Multiple copies could be located at the same or different genomic sites. The copies could be intact or truncated. Multiple copies at the same sites may be direct or

inverted tandem repeats. In addition, integration of the vector backbone sequence can occur (Assaad et al., 1993; Bauer et al., 1998; Dominguez et al., 2000; Fladung, 1999; Kononov et al., 1997). Complex integration patterns have been widely associated with gene silencing (Chandler and Vaucheret, 2001; Matzke et al., 1994; Muskens et al., 2000; Wassenegger, 2000).

Wide variation in the expression level of transgenes are frequently observed in independent transformants carrying transgenes involved in plant fatty acid synthesis (Dehesh et al., 1996; Hawkins and Kridl, 1998; Voelker et al., 1992; 1996, 1997). In. *B. napus*, the level of C12:0 in primary transformants carrying the bay-TE transgene varied from near zero up to 40% (Voelker et al., 1996). Among plants transformed with a soybean Δ9-desaturase gene, both over-expression and suppression of C18:0, targeted by the transgene, were obtained, with the C18:0 level ranging from 1.1% (below the 1.8% level of the non-transformed control plants) to 12.5% (in sense-suppressed plants) (Hitz et al., 1995).

Instability of transgene expression over generations is observed as reversion of expression states, i.e. inactivation to activation, and *vice versa*. In a population of *A. thaliana* plants transgenic for a hygromycin resistance gene (*hpt*), 50% of the plants failed to transmit the resistant trait to the progeny although the complete transgene was detected in all the plants. The tolerance was spontaneously restored in seedlings that originated from out-crosses with wild-type plants or with different sensitive transformants; but the expression was often lost again in the next generation (Scheid et al., 1991). High reversibility of the expression status, which cannot be explained by back mutation, characterizes the epigenetic nature of gene silencing (Matzke et al., 1994). Another example is with homozygous transgenic lines carrying the *npt* II and/or *hpt* genes. These lines, despite being homozygous, segregated continuously for generations, with plants ranging from fully resistant through intermediate to full sensitive in each population due to transgene silencing (Assaad et al., 1993).

The study by Assaad et al. (1993) also demonstrated that plants genetically identical for the transgene could have significantly different expression levels due to silencing. Plants of a maize line

homozygous for an aprotinin (a serine protease inhibitor) transgene accumulated near zero to high levels of the protein. The existence of multiple repeats, which is widely believed to be a possible factor inducing silencing, together with a wide variation in the mRNA level among individuals, suggested involvement of gene silencing (Zhong et al., 1999). In tobacco, a considerable variation was observed among DH lines developed from a single transformant originating from transformation of a haploid plant. Some DH line were highly resistant and others susceptible to the target potato virus Y (PVY) although each line had four copies of the same coat protein gene at the same loci. Putative host effect, associated with the ubiquitous but poorly defined products of "modifier genes", as well as gene silencing, could be the cause of the variation (Smith et al., 1994).

2.6.3 Transgene Silencing

Variation in the expression of transgenes is widely associated with gene silencing (Charrier et al., 2000; Meyer and Saedler, 1996; Scott et al., 1998). Gene silencing is referred to somatically or meiotically heritable repression of gene expression that is potentially reversible and is not due to mutation (Kaeppler et al., 2000).

Gene silencing can result from the blocking of transcription initiation (transcriptional gene silencing, TGS), or from the degradation of mRNA after transcription (post-transcriptional gene silencing, PTGS) (Chandler and Vaucheret, 2001; Matzke and Matzke, 1998; Wassenegger, 2000). Northern blot assays performed on cytoplasmic RNA in combination with run-on transcription, or RNase protection assays performed with isolated nuclei, can distinguish TGS from PTGS (Baulcombe, 1996; Kooter et al., 1999; Meins, 2000).

Although the distinction between TGS and PTGS is not always clear (Meins, 2000), TGS is associated with DNA methylation of the promoter region of the transgenes (Matzke and Matzke, 1998; Meins, 2000) probably in conjunction with a condensation of chromatin (Van-Blokland et al., 1997). PTGS is not necessarily correlated with methylation although in some cases it was associated with methylation in the coding regions (Fagard and Vaucheret, 2000; Meins, 2000).

In plants, PTGS is observed more often than TGS (Selker et al., 1999; Wasenegger, 2000). In comparison with TGS, which is frequently meiotically inherited despite having the reversibility (Meyer, 2000), PTGS is a less stable process and is more frequently reversible post-meiosis (Charrier et al., 2000; Fagard and Vaucheret, 2000; Meins, 2000). The initiation of PTGS is stochastic. PTGS usually occur only in a small proportion of lines transformed with any construct (Baulcombe, 1996). Also, only some plants in a genetically homogenous population show the silent phenotype (Meins, 2000). Later in the life-cycle, PTGS is usually lost post-meiotically and early in embryogenesis (Dehio and Schell, 1994; Kunz et al., 1996; Meins, 2000).

2.6.4 Factors Influencing Expression and Transgene Silencing

Many factors influence transgene expression and are involved in gene silencing, which include the number of transgene copies, structure of the inserts (e.g. direct or inverted tandem repeats, intact or truncated copy), presence of vector sequence, integration site in the genome, recipient genotype, environmental condition, presence of boundary elements or matrix attachment region (MAR), and infection of virus pathogens (Charrier et al., 2000; Dale et al., 1998; Maqbool and Christou, 1999). None of these factors alone can account for various cases of expression instability. The diversity in the variation of transgene expression may reflect interaction of these factors (Dale et al., 1998; Maqbool and Christou, 1999).

Copy number: Literature about the relationship between copy number and expression level of transgenes is conflicting (Hobbs et al., 1993; McCabe et al., 1999). A negative correlation was found in some studies (Cervera et al., 2000; Hobbs et al., 1993; Mannerlöf et al., 1997; McCabe et al., 1999). For example, transformants containing less copies of the glyphosate oxidase reductase gene (GOX) showed tolerance to higher doses of glyphosate, the active ingredient in Roundup(R), than transformants having multiple copies (Mannerlöf et al., 1997). Another detailed study provided an example for reduced expression levels with addition of extra copies (Matzke et al., 1989). In this study, two gene constructs were combined by performing sequential transformation on the same line. The first gene was switched off by the second gene. When the genes were segregated, the previously silenced gene was turned on. When the same two constructs were combined again in the same

genome, the silenced phenotype was returned.

Duplication of a transgene in plants could trigger gene silencing (Fagard and Vaucheret , 2000; Scheid et al., 1991). Homology-dependent gene silencing (HDGS) phenomenon, which is based on recognition of nucleic acid sequence homology between the interacting genes (Meyer and Saedler, 1996; Matzke et al., 1999), has been described in diverse organisms (Matzke et al., 1999). HDGS can occur due to the interaction between repeats located at the same site or different sites (Matzke et al., 1994). Analyses of various silenced and silencing transgenes showed that they often consist of multiple, tandem linked transgene copies. Several studies reported involvement of direct repeats, but many loci contain inverted repeats (Muskens et al., 2000).

However, there is insufficient evidence to conclude that multiple copies alone can necessarily lead to silencing. Firstly, a problem in studies with primary transformants is that, in independent transformants, the transgene is necessarily integrated at different genomic loci. As a result, the effect of extra copies is not easily separated from the influence of the integration positions (Assaad et al., 1993). In fact, wide variation among independent transformants, as well as the lack of correlation between copy number and expression level, were thought by some researchers to be caused mainly by positional effect (Allen et al., 2000; Gendloff et al., 1990)

Secondly, the effect of multiple copies could be confounded by influence from the structure of the inserts. Very few studies recloned and sequenced the transgene loci (Fagard and Vaucheret, 2000). Two studies clearly demonstrated inverted repeats inducing silencing (Mette et al., 1999; Morino et al., 1999).

Thirdly, a single transgene copy in the plant genome, based on sequencing of recloned inserts, can be silenced (Iglesias et al., 1997; Pröls and Meyer, 1992). PTGS of single-copy inserts occur even in haploid cells (Selker, 1999). On the other hand, reactivation of silenced genes can occur without reduction in the copy number as a prerequisite (Scheid et al., 1991). Thus, the existence of multiple copies is not a necessary requirement for gene silencing.

Fourthly, the presence of multiple copies does not always lead to repression of expression although it is likely to do so (Hobbs et al., 1993). In non-transformed plants, neither single allelic pairs nor repeated gene families are normally silenced (Assaad et al., 1993). In transformation experiments, introduction of extra copies of a plant endogenous gene usually leads to co-suppression in only a limited number of primary plants (van Blokland et al., 1994; Matzke et al., 1994). Some reports have showed a lack of correlation between transgene copy number and the ability to suppress endogenous genes (van der Krol et al., 1990). On the contrary, over-expression can be obtained by introducing extra copies (Matzke et al., 1994). Among canola plants transformed with homologous genes coding for Δ12- or Δ15- desaturases, both co-suppressed and over-expressed plants were obtained (Hitz et al., 1995). Moreover, a neutral correlation between copy number and expression, has been observed with various transgenic plants (Bauer et al., 1998; Hobbs et al., 1993; McCabe et al., 1999). These reports support that other factors could be involved in the silencing events where multiple copies were present.

In addition, a positive correlation between copy number and expression level has been widely documented (Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999; van der Hoeven et al., 1994). For example, transgenic tobacco plants showed an increased expression level in general as the copy number of the chloramphenicol acetyl transferase (CAT) transgene increased from one to four, although some plants with one copy produced more CAT than plants with more copies (Gendloff et al., 1990). Relatively less influence from integrational position than from copy number on the variation in this population (Gendloff et al., 1990), could be the main reason for the positive correlation.

In many cases where there is no consistent positive correlation, multiple copies are robustly expressed in most of the transgenic plants (Blundy et al., 1991; McCabe et al., 1999; Que et al., 1997). For instance, transgenic potato plants carrying the GUS gene did not show a general positive correlation, but the plants with the highest expression level always contained multiple copies (Blundy et al., 1991). A transgenic maize line with a high expression level of the aprotinin transgene contained at least 20 copies (Zhong et al., 1999). Among *B. napus* plants carrying the bay-TE

transgene, transformants with the highest C12:0 levels all had multiple copies (up to approximately 15) either at multiple loci or in a single locus (Voelker et al., 1996). Similarly, a positive effect of multiple copies was demonstrated with a construct containing two copies of the bay-TE gene. Plants transformed with this construct showed an average C12:0 level up to 2-fold higher than plants transformed with the same plasmid but having only one copy of the bay-TE gene in the construct (Voelker et al., 1996).

Positive correlation obtained with transgene plants from independent transformation events could be due to gene dosage effect (the more copies, the higher expression level), or other reasons, e.g. a higher probability for plants carrying multiple copies to have copies in favorable genomic positions than plants having a single T-DNA insert (Wolters et al., 1998).

The existence of dosage effect of transgene copies is demonstrated by comparing hemizygous and homozygous transgenic plants, where homozygotes are expected to have double the copy number of the hemizygotes (Azhakanandam et al., 2000; Beaujean et al., 1998; Hobbs et al., 1993; Tenllado and Diaz Ruiz, 1999). Transgenic tobacco F_1 plants that originated from cross-fertilization between two different homozygous transformants with a high expression level, showed an expression level that closely equalled the parents. Individual F_2 plants expressed the transgenes at 50%, 100%, 150% and 200% of parent values as the copy number in the whole genome increased from one to four copies, whether the copies were allelic or non-allelic (Hobbs et al., 1993). In another study, comparison of haploid plants with the corresponding DH plants also showed dosage effect (Beaujean et al., 1998).

Structure of T-DNA Inserts: Complex T-DNA inserts, including direct or inverted tandem repeats, truncated copies, arrangement of transformed sequences in an insert, are often associated with gene silencing and variation in transgene expression (Fagard and Vaucheret, 2000; Hobbs et al., 1990; Mittelsten et al., 1998; Muskens et al., 2000). Integration of vector backbone sequences has a negative effect on the expression (Fu et al., 2000; Jakowitsch et al., 1999).

Tandem linked T-DNA can be arranged as direct repeats, right-border inverted repeats (IR) or left-border IRs, or a combination of the three (Muskens et al., 2000). Silencing is more likely to occur when transgene copies are tandem repeated compared to unlinked (Allen et al., 2000; Garrick et al., 1998).

Inverted repeats (IRs) are particularly potent silencers for transgenes and endogenous genes (Fagard and Vaucheret , 2000; Muskens et al., 2000; Selker, 1999). Stam et al. (1998) observed that all petunia plants showing post-transcriptional gene silencing carried an inverted repeat arrangement. Hobbs et al. (1993) found with transgenic tobacco plants that inverted repeats gave low expression, while single T-DNA inserts gave high expression regardless the number of single-copy inserts; plants carrying both a high expressing single-copy insert and an inverted repeat showed totally or partially suppressed expression.

IRs can be involved in both PTGS and TGS (Hamilton et al., 1998; Muskens et al., 2000; Selker, 1999). The potential of IRs to produce double-stranded (ds) RNA might be the key to the both modes of gene silencing (Muskens et al., 2000). DsRNA have a dural role of triggering *de novo* methylation by RNA-DNA paring and inducing sequence-specific degradation (Fire, 1999; Kooter et al., 1999; Muskens et al., 2000; Selker, 1999).

Truncated T-DNA copies without either the left side or the right side have been detected in transformed plants (Dominguez et al., 2000; Fladung, 1999; McCabe et al., 1999). Truncated transgenes are capable of generating incomplete transcripts, leading to the formation of aberrant RNA (aRNA) species, which induce gene silencing (Kohli et al., 1999). In a Mexican lime (*Citrus aurantifolia* Swing.) transformation experiment, incomplete T-DNA copies without the left border co-existed with intact copies in up to 70% of the transformants, and no correlation was observed between copy number and expression level in these plants (Dominguez, et al., 2000). Reduced expression of endogenous genes has also been reported after introduction of incomplete homologous sequences (Goring et al., 1990; Hamada and Spanu, 1998; Hobbs et al., 1993). If the existence of truncated copies was a cause for the variation in the expression, reduction or elimination of

incomplete copies could possibly improve the relationship between copy number and expression level. As expected, transformation with a GUS gene flanked by two selectable markers did show less variation (Bhattacharyya et al., 1994).

It has been demonstrated, with a series of alleles located in the same genomic locus, that arrangement of the sequences in an insert and the combination of alleles in the two chromosome homologs influence transgene expression (Assaad et al., 1993). The multiple alleles were produced by homologous recombinations of a primary transgenic allele that contained a hpt (hygromycin phosphotransferase) gene flanked by two different non-overlapping deletion mutant npt II (neomycin phosphotransferase II) genes. Each of the three genes were flanked with a copy of the 35S promoter and a copy of nopline synthase nos3' terminator for expression. The alleles included one allele containing only the intact npt II gene and the regulatory sequences, and the other alleles containing, in different arrangements, the intact npt II gene, a npt II deletion gene, with or without the hpt gene. Different alleles, as well as different allelic combinations of these alleles in the two homologs, produced significantly different expression levels (Assaad et al., 1993).

However, complex T-DNA integration pattens are not always correlated with low expression (Hobbs et al., 1990; McCabe et al., 1999). Many repeated transgenes were well expressed, including genes in IRs (Luff et al., 1999; Jones et al., 1987). Also, tandem repeated endogenous genes such as rRNA, tRNA and histone genes, are normally expressed (Muskens et al., 2000). In addition, high-level expression was observed with multiple inserts including incomplete copies (Hobbs et al., 1990).

Genomic Position: Since foreign DNA integrates almost exclusively at random, non-homologous sites during transformation (Allen et al., 2000; Mengiste and Paszkowski, 1999), some integrations may occur in transcriptionally active chromatin environments, others in condensed, transcriptionally inert chromatin regions (Allen et al., 2000). It is believed that transgenes in heterochromatic areas such as surrounding centromeres are prone to silencing and give rise to reduced and/or variable expression (Allen et al., 2000; Maqbool and Christou, 1999; Weiler and Wakimoto, 1995). The repression of transgene expression in such areas could occur due to alteration of chromatin structure,

caused by repressive chromatin spreading from adjacent surrounding sequences (Chandler and Vaucheret, 2001; Fagard and Vaucheret, 2000). Thus, this phenomena can affect transgenes present even as a single copy (Fagard and Vaucheret, 2000). Conversely, transgenes integrated into subtelomeric regions may be strongly expressed (Topping et al., 1991). Besides, variation in transgene expression can be caused by the integration of transgenes into positions adjacent to endogenous regulatory elements such as enhancers (Allen, et al., 1996, 2000). Moreover, a local discrepancy at the junction between the transgene and the surrounding sequences, e.g. different GC content or existence of the vector sequences, may disorganize chromatin structure and destabilize gene expression (Fagard and Vaucheret, 2000).

Wide variation among independent transformants, as well as the lack of positive correlation between copy number and expression level, could be mainly, or entirely, due to positional effects (Allen et al., 2000; De Neve, 1999; Gendloff et al., 1990). *B. napus* transgenic plants carrying a fixed number of the bay-TE gene at different loci showed a wide range of the C12:0 levels; e.g., plants with one copy varied from zero to 16%, and plants with two copies varied from 2% to 27%, indicating substantial influence of chromosomal location on the bay-TE expression (Voelker et al., 1996). In some studies, however, no significant positional effect was detected (Gendloff et al., 1990; Hobbs et al., 1990, 1993).

Recipient Genotype: The literature contains several terms to describe the genetic factors in the host genome on the expression of transgenes, such as 'host component', 'modifier genes'(Smith et al., 1994), 'background genotype' (Dale et al., 1998; Sachs et al., 1998), in addition to 'genetic background' (Dale et al., 1998; Fearing et al., 1997).

The effect of genetic background has been well described in classical genetic studies with non-transgenic plants (Dale et al., 1998), and has also been reported in transgene studies. In a genetic study of the C18:3 level in *B. napus*, the variation which could not be fully accounted for by major genes was attributed to the existence of 'minor genes' in the plant genome (Jourdren et al., 1996). It is also observed that the C22:1 level in *Brassica* is influenced by minor genes (Browse and

Somerville, 1994). In soybean, wide variation (15 to 30 g/kg) in the C16:0 level in the progeny homozygous for the alleles determining either the reduced or the normal C16:0 level, was explained by an undetermined number of 'genetic modifiers'(Rebetzke et al., 1998).

Transgenes have been transferred between genotypes by crossing (Dale et al., 1998). A study with a white clover line (*Trifolium repens* L.) carrying a GUS gene showed a four-fold variation in the GUS activity within the lines even though each plant contained a copy of the same T-DNA insert (Scott et al., 1998). Progeny from plants with high expression levels showed higher GUS activity than progeny from plants with low expression levels, suggesting the influence of background genotype on the expression.

The genotype of host plants influences the expression in various mechanisms. In potato, the expression level of a GUS gene driven by the promoter of a potato tuber soluble protein (patatin) gene was five-fold higher in cultivar 'Desiree' than in 'Maris Bard', whereas, with another promoter, the expression in the two cultivars was not significantly different (Blundy et al., 1991). Possible differences in the transcription factors between the two genotypes was proposed as an explanation. Co-evolution of transcription factors and promoters has been reported (Dover and Flavell, 1984; Miesfeld and Arnheim, 1984; Blundy et a l., 1991). The influence from genetic background could also result from the action of the host plant genes which have a similar phenotype as the transgene. Transgenic tobacco lines carrying the tobacco vein mottling virus (TVMV) coat protein (CP) gene showed different levels of resistance against the target viruses depending on the recipient genotypes; the levels were not only dependent on the presence of an endogenous plant host resistance gene VAM but also closely followed the difference in the tolerance of the non-transgenic recipient genotypes (Xu et al., 1997).

Interactions between transgene and endogenous, homologous sequences in the background genotype is another possible factor leading to differential expression in different genetic backgrounds (Dale et al., 1998). RNA-directed DNA methylation (RdDM), for example, is very sensitive in terms of the minimal DNA sequence that is required as a target. Approximately a 60 bp of identical sequence

(Sijen et al., 1996), or a 60-70% sequence identity (Angenent et al., 1993; Kunz, 1997), is sufficient to trigger PTGS. Thus, if sequences homologous to the transgene exist and are polymorphic in a transgenic population, variation in the expression could occur due to the interactions between the transgene and the endogenous sequences. This possibility was observed in transgenic potato (*Solanum tuberosum*) , which is a heterozygous polyploid species and has a series of granule-bound starch synthase (GBSS1) alleles (Wolters et al., 1998). The antisense effect of a GBSS1 gene on amylose production ranged from complete suppression to no discernable inhibition in different transformants, depending on the composition of endogenous GBSS1 alleles in the plants since a GBSSI allele that is more effective than other alleles may be more difficult to suppress.

More directly, silencing and variation can be regulated by special plant genes involved in the induction and release process of silencing. Mutants defective in PTGS and mutants showing increased PTGS have been identified in plants (Dehio and Schell, 1994; Elmayan et al., 1998). Methylation of the multigene *PAl* family in *Arabidopsis* requires DDMA and DDM2 genes (Fagard and Vaucheret, 2000). Repetitive sequences (RPS) exist in the genome of most plant species and are often methylated (Fagard and Vaucheret, 2000). Transformation with a construct containing a 1.6-kb RPS from *Petunia hybrida* led to more variable 35S-GUS expression in petunia and tobacco plants (ten Lohuis et al., 1995). Methylated RPS elements may recruit chromatin components that induce TGS of neighboring transgenes (Fagard and Vaucheret, 2000).

In plant fatty acid biosynthesis, some components are encoded by multiple genes, which allows fine-tuning of fatty acid level. In *Arabidopsis*, ACP and the 18:0-ACP desaturase are each encoded by at least five genes, although some other enzymes are encoded by a single gene (Browse and Somerville, 1994). In addition, each member in the multiple gene family can have more than one copy and be polymorphic. In *B. napus*, Δ12- and Δ15- desaturases are each encoded by 4-6 and 6-8 copies per haploid genome, respectively (Scheffler et al., 1997). In sunflower, examination of desaturase genes with eight inbred lines showed that the length and nucleotide sequence of introns, as well as the 5'-untranslated region are polymorphic (Hongtrakful et al., 1998). In safflower, two C18:0-ACP cDNA TE isoforms showed similar but not identical specificity, with one being less

discriminating against C16:0- and C18:0-ACP than the other although both prefer C18:1-ACP (Knutzon et al., 1992). Thus, it is possible that polymorphism in fatty acid genes in the host genotypes contributes to the variation in the fatty acid composition in transgenic plants engineered with fatty acid genes.

In some studies, no significant genetic background effect was observed. Several maize lines carrying the CryIA(b) transgene did not show significantly different levels of the CryIA(b) protein although these lines had different parentage and had a wide variation within the lines (Fearing et al., 1997). In *Brassica*, a line containing a single insert of the bar gene was crossed with a range of ecotypes of five *Brassica* species. The observed segregation ratios in F_1 varied from one species to another, but the expression in the genetic backgrounds tested was similar to that in the *B. napus* plants (Dale et al., 1998).

Reciprocal Crossing: Generally, the inheritance of transgene expression was not influenced by transmission of a gene through either the male or female parent in reciprocal crosses although examples of parental bias exist (Koltunow and Brennan, 1998; Meyer et al., 1993). Influence of maternal plant on the expression of a RNase transgene in the seed was reported in tobacco (Koltunow and Brennan, 1998). A study in the T-2 generation of *Lotus corniculatus* lines transgenic for the GUS genes showed that transgenic plants as female or male parents led to different frequencies of plants expressing the transgene in the offspring (Schröder-Pontoppidan et al., 2000)

Growth Environmental Influence: Growth conditions were repeatedly observed to influence the expression of transgenes (Matzke et al., 1994; McCabe et al., 1999; Senior, 1998). Expression may be affected by increased temperature (Conner et al., 1998; Köhne et al., 1998; Matzke et al., 1994) and high light intensity (van der Krol et al., 1990). A study reported that kanamycin-sensitive progeny from self-pollination of homologous 1-locus tobacco transgenic lines occurred at a frequency of 0.5-5.9 x 10^{-5} under close-to-optimum environmental conditions, but the frequency became as high as 1.5-3.8 x 10^{-3} under heat and/or drought stress (Conner et al., 1998). Another study indicated different heat stability of sequences. A GC rich sequence showed reduced expression

while the expression of an AT rich sequence was stable under the same heat stress although the two sequences coded for essentially the same protein (Köhne et al., 1998).

In addition, *in vitro* culture could influence transgene expression. During *in vitro* culture, DNA methylation may occur (Brown et al., 1990; Kaeppler and Phillips, 1993; Olhoft, 1996), which could condition chromatin structures and genetic mutations, thus leading to variation in the expression (Kaeppler et al., 2000). Variation due to epigenetic silencing events in culture has been observed (Kaeppler et al., 2000). However, in an experiment with bay-TE transgenic lines, the influence of somaclonal variation on the expression was discounted since the level of C12:0 targeted by the TE transgene remained the same as that in the original transgenic lines following re-transformation of the transgenic plants to introduce extra copies (Voelker et al., 1996).

2.6.5 Mechanisms of Gene Silencing

Transcriptional Gene Silencing: TGS can be induced by: 1) the surrounding heterochromatin; 2) endogenous sequences, e.g. repetitive sequences (ten Lohuis et al., 1995), 3) local discrepancy at the junctions between the transgene and the genomic sequence, as represented by significant difference in the GC content; 4) multiple copies, especially if they include truncated copies, direct or inverted tandem repeats, or particular spatial arrangement of the sequence in the integration site (Assaasd et al., 1993; Fagard and Vaucheret, 2000; Mette et al., 1999).

In all cases, transgenes that are involved in TGS showed hypermethylation, and where tested, chromatin condensation was observed (Fagard and Vaucheret, 2000). DNA methylation is a modification of bases by addition of a methyl group either to the cyclic carbon in C5-methylcytosine, or to non-cyclic nitrogen residues of adenine (N6-adenine) or cytosine (N4-cytosine). Methylation is catalyzed by enzymes called DNA methyltransferases (Finnegan and Kovac, 2000). In plants, the association of hypermethylation to transgene silencing is based on analyses of transgenes with reduced expression (Amasino et al., 1984; Wassenegger, 2000). Also, the association is supported by results with T-DNA demethylation, which can lead to the restoration of the expression of silenced genes (Ooms, 1992). There is an increasing evidence that DNA methylation initiates TGS

(Kooter et al., 1999; Mette et al., 1999; Nan et al., 1998, Ng and Bird, 1999; Wassenegger, 2000).

Methylation events can be divided into two categories: DNA-mediated methylation (DmDM) or RNA-directed methylation (RdDM) (Wassenegger, 2000). DmDM can be triggered by 1) pairing of homologous DNA sequences; 2) condensed chromatin structure in the integration sites; 3) inserts with a special structure, e.g. inverted repeats; 4) recognition of sequences with repeated CG or CpNpG within particular sequence context (Muskens et al., 2000; Stam et al., 1998; Wassenegger, 2000)

RdDM represents a general mechanism for the induction of *de novo* methylation in plants (Mette et al., 1999; Jones et al., 1998). Double-stranded (ds) RNA seems to be important in RdDM (Fire et al., 1999; Wassenegger, 2000). dsRNA can be produced due to the existence of a promoter located downstream of, or within, a gene whenever transcription is driven in an opposite direction. Subsequent annealing of antisense RNA (asRNA) and sense RNA would lead to dsRNA. Alternatively, dsRNA can originate from the transcription of inverted repeats (Wassenegger, 2000). A third approach is based on RNA-dependent RNA polymerase (RdRP), which is capable of transcribing complementary RNA from RNA molecules (Schiebel, et al., 1998). The templates for RdRP can result from irregular termination of transcription, irregular RNA processing, and mRNA degradation products. In addition, RNA with a double-stranded structure can be formed if complementary regions exist, as in transcripts from some IRs (Wassenegger, 2000). For RdMD, RNA is assumed to bind to DNA which is complementary to the directing RNA. The binding leads to DNA methylation along the RNA-DNA duplex (Wassenegger, 2000). However, the putative enzyme, *de novo* methyltransferase (MTase), including MTase recognizing RNA-DNA hybrids, have not yet been identified (Finnegan and Kovac, 2000; Wassenegger, 2000).

Expression repression of methylated transgenes is probably caused by interference of methylation with transcription factor binding (Meyer, 2000). There is evidence for such a possibility (Ammerpohl et al., 1998; Pikaart et al., 1998). Alternatively, methylation could repress the expression via remodeling chromatin state: methylated transgenes possibly recruit histone

deacetylases via a methyl-cytosine-binding protein (e.g. MeCP2); the binding then initiates chromatin condensation via deacetylation, finally blocking the transcription process (Fagard and Vaucheret 2000; Li, 1999; Meyer, 2000). It is believed that the availability of the binding site for RNA polymerase and transcription factors is determined by the local chromatin structure (Allen et al., 2000; Meyer, 2000).

However, methylation is not a necessary preclude for repressive chromatin state and TGS (Chandler and Vaucheret, 2001; Meyer, 2000; Muskens et al., 2000). In methylation-deficient *Arabidopsis* mutants and plants in which the DNA methyltransferase activity is inhibited by antisense RNA, some TGS silenced (trans)genes were not reactivated (Mittelsten et al., 1998). Two nuclear proteins, DDM1 and MOM1, involved in TGS, have been identified. Impairment of DDM1 can release both TGS and methylation of transgene arrays (Chandler and Vaucheret, 2001); but impairment of MOM1 can release TGS but not methylation (Amedeo et al., 2000). These studies suggest that TGS can be methylation dependent or independent. Thus, in some silencing events, methylation could be a consequence of the repressive chromatin state (Fagard and Vaucheret, 2000).

Post-Transcriptional Gene Silencing: PTGS is characterized by post-transcriptional RNA degradation in a sequence-specific manner, which can propagate systematically throughout the plant (Baulcombe, 1996; Fagard and Vaucheret, 2000). PTGS is observed in a number of situations: 1) single copy T-DNA that is transcribed can be silenced; 2) transcribed complex T-DNA inserts, particularly those containing inverted repeats or backbone sequence, can induce PTGS; 3) transcriptionally silenced transgenes, promoterless transgenes, and fragments of DNA homologous to resident transgenes, which presumably are not transcribed or only weakly transcribed, can trigger PTGS of high-expressing target genes; 4) down-regulation of an endogenous gene can be efficiently achieved using the antisense transgene, transcribed or untranscribed (Que et al., 1997; Stam et al., 1998; van Blokland et al., 1994).

Numerous models have been proposed to explain PTGS. None can fully explain the broad range of the silencing phenomena (Meins, 2000). RNA threshold models hold that cells have a negative

autoregulatory mechanism for sensing transcripts similar in the sequence (Allen et al., 2000; Lindbo et al., 1993; Smith et al., 1994). When the combined concentration of these transcripts exceeds a critical level, the degradation system is activated. These models predict dependence of PTGS on the transcription and provide an explanation for the silencing triggered by high copy numbers. However, they could not explain why the expression of silenced transgenes can be reduced to very low levels rather than to just below a threshold level (Meins, 2000).

Most current models assume that silencing is triggered by special signals such as asRNA or dsRNA, rather than by a certain critical level of the transcripts. The signals are thought to interact with target RNA in a sequence-specific fashion and then result in the degradation of RNA, usually in the cytoplasm (Meins, 2000). asRNA has been detected in PTGS plants in several different experiments (Hamilton and Baulcombe, 1999; Morino et al., 1999). Accumulation of 25-nucleotide long asRNAs was strictly correlated with PTGS in several different experimental systems (Hamilton and Baulcombe, 1999). dsRNA can be produced in a number of ways (Fagard and Vacheret, 2000; Schiebel et al., 1998), as mentioned previously.

Degradation of asRNA and dsRNA may involve both exonucleolytic cleavage and endonucleolytic pathways (Meins, 2000). In plants, local double-stranded sequences might serve as substrates for dsRNA-specific RNase (dsRNase); or, asRNA and dsRNA attract ssRNase for degradation (Fire, 1999; Metzlaff et al., 1997; Waterhouse et al., 1998).

The stability, reversibility, and diversity of PTGS phenotypes can be well explained by a modified biochemical-switch model (Meins and Kunz, 1995; Meins, 2000), with the incorporation of the asRNA and dsRNA signals (Meins, 2000). Based on this model, the production of the signals becomes sustained, thus maintaining the silent state, when the concentration of the signals from the silencing loci, from other cells, and from the degradation of the target RNA, exceeds a critical threshold (Holtorf et al., 1999; Meins, 2000). The sustaining may be due to self-replication triggered by the signals above the threshold level and/or by the degradation. The influence of environmental, developmental, and physiolocal factors on the transcribed silencing loci, on the target genes, and on

the cell-to-cell movement of the signals leads to a diversity of silencing phenomena. When the concentration is near the critical threshold, transient fluctuations in the target RNA content or signal content cause stochastic patterns of the silencing. But there is no direct demonstration that asRNA, dsRNA or other aberrant RNAs (aRNAs) generated in silent tissues can induce silencing in biological assays (Meins, 2000).

Methylation has also been associated with PTGS (Fagard and Vaucheret , 2000; Ingelbrecht et al., 1994; Meins, 2000; Wassenegger, 2000); however, its role in PTGS is unclear (Wassenegger, 2000). It is possible that the transcription of methylated coding regions could produce aRAN molecules (Fagard and Vaucheret, 2000). Since methylation-deficient organisms, e.g. *Neurospora crassa*, also display PTGS-like process, methylation seems not essential for PTGS (Cogoni et al., 1996; Wassenegger, 2000).

2.7 Doubled Haploid Lines and Related Variation

2.7.1 Doubled Haploid Line Development

In *Brassica*, doubled haploid (DH) plants are developed through *in vitro* culture of microspores (immature pollen grains), followed by regeneration and chromosome doubling (Ferrie and Keller, 1995). This technology is based on a breakthrough discovery reported by Lichter et al. (1982) in which a large number of haploid embryos developed when immature pollen were cultured in isolation from anthers. Embryogenesis is induced through a heat treatment of the isolated microspores in media (Heberle-Bors, 1999; Pechan, et al., 1991; Touraev, et al., 1996a, b, 1997). Subsequent culture of the haploid embryos in appropriate media results in the regeneration of haploid plantlets. Doubling of the chromosome number in the haploid plants is usually achieved by treating the plants with colchicine (Gland, 1981; Ferrie and Keller, 1995). Alternatively, colchicine can be added *in vitro* into the media immediately after the isolation of microspores for the doubling purpose, which saves time and labor compared to the root treatment (Mathias and Robbelen, 1991; Möllers et al., 1994), and greatly increases the efficiency of doubling (Möllers et al., 1994). Colchicine in the induction media can improve embryogenesis and has no negative effect on the further development of the embryos (Zaki and Dickinson, 1991; Zhao, et al., 1996).

2.7.2 Utilization of DH Lines

For plant breeding, complete homozygosity can be realized in a single generation with the DH technology, thus avoiding repeated generations of inbreeding practice for the development of pure breeding lines (Chen and Beversdorf, 1990; Ferrie and Keller, 1995; Finnie et al., 1991; Wenzel and Foroughi-wehr, 1994). An additional advantage of this technology is to facilitate plant selection in the absence of dominance-related variation and within-family segregation (Finnie et al., 1991). In DH populations, the phenotypic effect of a recessive allele is not masked by the dominant allele, thus the genetic segregation is greatly simplified compared to an inbred population originating from hybrids. For example, an 1:1 segregation ratio is expected for a single gene in DH populations rather than a 3:1 ratio for a F_2 population. This facilitates the identification of plants having desirable recessive traits for plant breeders with a smaller plant population (Ferrie and Keller, 1995; Stringam, 1999). In addition, intermediate phenotypic expression resulting from intragenic/allelic interaction is excluded in DH populations. This is of particular benefit in some genetic studies, e.g. in genetic mapping and QTL (quantitative trait loci) analysis (Kasha and Kleinhofs, 1993; Wenzel and Foroughi-wehr, 1994; Wenzel, 1995). Because of these advantages, DH technology has been incorporated into canola breeding procedures and resulted in the development of superior cultivars and useful breeding lines (Friesen and Scarth, 1999; Stringam et al., 1995). DH technology has also been used in breeding for a number of other crops (Hu and Kasha, 1997). In addition, DH plants are used in studies of inheritance patterns of plant characters, effects of specific genes, genetic polymorphism identification, and genetic mapping (Ferrie and Keller, 1995; Pickering and Devaux, 1992).

2.7.3 Variation Associated with DH Line Development

The DH line development procedure includes an *in vitro* culture process and chromosome doubling treatment (Ferrie and Keller, 1995). Both *in vitro* culture and chromosome doubling treatment can cause phenotypic variation (Kaeppler et al., 2000; Niemirowicz-Szczytt, 1997).

Variation among plants from somatic tissue culture and microspore culture has been well documented (Evans, 1989; Kaeppler et al., 2000). The variation among plants from somatic tissue

culture is referred to as somaclonal variation (Kaeppler et al., 2000), whereas the term gametoclonal variation is used to describe the variation caused by microspore culture process (Evans et al., 1984 Pickering and Devaux, 1992). An important difference between gametoclonal and somaclonal variation is that single-gene mutations induced in somatic cell culture would be in heterozygous conditions in the regenerated plants and will segregate (Evans et al., 1984).

In plants, somaclonal variations are detected by high frequencies of qualitative mutations and quantitative variations among progeny of plants that are expected to be genetically identical (Kaeppler et al., 2000). A study with maize culture grown for eight months found that, on average, every regenerated plant contained 1.32 mutants that produced a visible phenotype (Lee and Phillips, 1987). Quantitative variation in many traits, e.g. plant height, plants biomass, grain yield, and agronomic performance, has been reported, and genetic studies indicate alterations of numerous loci (Kaeppler et al., 2000). In general, the longer the culture phase, the higher the level of variation induced (Dale and Irwin, 1994; Jorgensen and Andersen, 1989; Karp, 1989).

In *B. napus*, DH lines exhibited a wider variation in agronomic performance, e.g. days to flower, than plants from single-seed descent derived from the same hybrid plants (Stringam, 1999). In another study, variation in the fatty acid composition was increased after tissue culture and transformation compared to plants derived from seeds. The C18:1 level in the seed oil ranged from 39-72% among transformants, 47-76% among tissue culture-derived plants, and 55-69% among seed-derived plants (Schröder-Pontoppidan et al., 2000).

In wheat, gametoclonal variation was detected based on analyses of agronomic performance, isozyme and karyotype of DH lines (Baenzier et al., 1989; Marburger and Jauhar, 1989). In tobacco, considerable variation was observed among DH lines from a single transformant originating from transformation of a haploid plant. Some lines were highly resistant and others susceptible to the target potato virus Y (PVY) although each line had four copies of the same coat protein gene at the same loci. The putative host effect associated with the ubiquitous but poorly defined products of "modifier genes", as well as gene silencing was considered to be the causes (Smith et al., 1994). In

rice, gametoclonal variation in agronomic and physiological traits were reported, e.g. in heading date, seed fertility, plant height and chlorophyll content (Oono, 1981).

Colchicine treatment has long been known to induce mutation (Franzke and Rose, 1952) at the chromosomal structural level and DNA sequence modifications (Niemirowicz-Szczytt, 1997; Francis and Jones, 1989). Since colchicine treatment is involved in the DH line development process, some of the observed variation between DH lines may be due to the mutagenic effect of colchicine (Evans et al., 1984). In wheat, Snape et al. (1988) reported that 'gametoclonal variation' among DH line plants was probably the results of the colchicine treatment. In a study on *B. napus*, the frequency of tetraploids varied from very low to more than 6% with the optimum *in vitro* colchicine treatment, depending on the genotype of the donor plants (Möllers et al., 1994).

However, in some studies no increased variation or instability was detected among plants that regenerated from culture, with or without application of colchicine treatment. In barley, Finnie et al. (1991) did not find gross chromosome abnormalities in chromosome number and chromosome structure among DH plants. No difference was detected between DH lines and the donor plants, and between DH lines for which the chromosome number was doubled by colchicine treatment and DH lines for which the chromosome number was spontaneously doubled, by gel electrophoresis analyses of a storage protein and five isozymes and by RFLP analysis with one enzyme and four DNA probes. Pickering and Devaux (1992) did not observe gametoclonal variation among DH lines developed from an inbred donor, and thought that such variation can be disregarded in barley breeding and research. In wheat, Hu and Kasha (1997) found that only 1.7% of the haploid plants cultured from microspores were aneuploids, only four of more than 100 DH lines showed visible mutations, and most DH lines were similar to the donor in field-measured traits, e.g. yield, 1000-kernel weight. In *B. napus*, somaclonal variation was discounted in a re-transformation experiment with plants already harboring the *UcFatB1* gene in order to introduce extra copies of the gene, since the level of C12:0, targeted by the gene, remained the same as that of the original transgenic lines (Voelker et al., 1996).

It should be emphasized that the progeny of most DH lines are genetically uniform and homogenous.

Studies on the genetic stability of DH progenies in wheat, barley, rice and corn showed that about 90% of the DH lines were genetically uniform (Hu and Kasha, 1997). With regard to *B. napus*, the DH cultivar 'Quantum' is very uniform in erucic acid and glucosinolate contents, and it displays no visual shifts in the appearance over generations (Stringam, 1995).

2.7.4 Genetic Basis of the Variation

Variation in phenotype is determined by genetic or epigenetic factors (Kaeppler et al., 2000). The nature of somaclonal variation includes karyotype changes, sequence modifications (single gene mutations), or epigenetic modifications (Dale and Irwin, 1994; Kaeppler et al., 2000). Karyotype mutations, e.g. alterations in chromosome structure and chromosome number, are prevalent in culture. Single gene mutations are most likely due to base changes, mainly point mutations or very small insertion /deletions (Kaeppler et al., 2000). In two studies with maize mutants, the mutant alleles have been linked to A-to-T transversions (Brettell et al., 1986; Dennis et al., 1987). Epigenetic control of gene expression is defined as a somatically or meiotically heritable alteration in gene expression that is potentially reversible and is not due to DNA sequence modification (Kaeppler et al., 2000). Somaclonal variations of epigenetic nature, such as changes in the methylation pattern, occurs frequently in culture (Brown et al., 1991; Kaeppler and Phillips, 1993; Kaeppler et al., 2000; Olhoft, 1996). The methylation pattern can differ among regenerated plants. Methylation alters chromatin structure, which is hypothesized to be an underlying mechanism for the high frequency of quantitative phenotypic variation via modulating the effects of multiple loci. Methylation is also associated with cytological changes and DNA base changes (Kaeppler et al., 2000).

CHAPTER 3

EFFECT OF RECIPIENT GENOTYPE ON THE LEVELS OF THE FATTY ACIDS TARGETED BY ACYL-ACP THIOESTERASE TRANSGENES IN *BRASSICA NAPUS*

3.1 ABSTRACT

Brassica napus lines transformed with foreign acyl-acyl carrier protein (ACP) thioesterase (TE) genes accumulating lauric acid (C12:0) or enhanced levels of palmitic acid (C16:0) in the seed oil were available for study. Four transgenic parental lines, individually transformed with genes coding for the bay-TE (Ua FatB1), elm-TE (Uc FatB1), nutmeg-TE (Mf FatB1) and cuphea-TE (Ch FatB1), were crossed reciprocally with three non-transgenic cultivars to produce transgenic F_1 seeds. The three cultivars have distinct fatty acid profiles typical of conventional rapeseed cultivars with a high erucic acid (C22:1) level and canola cultivars with a low C22:1 level and a high or low linolenic (C18:3) level. DH lines were developed from the F_1 generation of the crosses. The influence of recipient genotype, cytoplasm and maternal parent on the target fatty acids of the TE transgenes was investigated by analysis of the fatty acid composition of the transgenic F_1 seeds and DH lines. The substrate specificity of the TE enzymess is not significantly influenced by the recipient genotype, with C12:0 being the major target fatty acid for the bay-TE and C16:0 being the major target fatty acid for the other three TE transgenes in DH lines from different crosses. The expression of the TE was not different in recipient genotypes with lower linolenic acid (C18:3) levels from the expression in recipient genotypes with higher C18:3 levels. However, the expression in low erucic acid (C22:1) genotypes resulted in significantly higher levels of the target fatty acids than the expression in high C22:1 genotypes. Maternal effects on the levels of the target fatty acids were evident between reciprocal F_1 seeds from five of eight pairs of reciprocal crosses between the transgenic parents and two of the three cultivars, AC Excel and Mercury. Cytoplasmic effects were not detected since the transgenic DH lines from the reciprocal crosses were not significantly different in the levels of the target fatty acids. These results implied that it is important to use appropriate recipient genotypes

to obtain an optimum level of the target fatty acids. In addition, co-expression of two different TE transgenes in the same seeds was observed, providing an additional approach to develop *B. napus* cultivars with modified fatty acid compositions.

Abbreviations: C12:0, lauric acid; C16:0, palmitic acid; C18:3, linolenic acid; C22:1, erucic acid; DH, doubled haploid; TE, acyl-acyl carrier protein (ACP) thioesterase; MCFA, medium-chain fatty acids.

3.2 INTRODUCTION

Conventional 'canola' cultivars (*B. napus* L.) have a seed oil fatty acid composition predominantly comprised of fatty acids with a chain length of 18 carbon atoms, with only ca. 4% palmitic acid (C16:0) and traces of saturated medium-chain fatty acids (MCFA), C8:0 - C14:0 (Eskin et al., 1996; Voelker et al., 1996). Plant oils rich in MCFA have a number of uses in food and non-food industries (Martini et al., 1995; Ohlrogge, 1994; Töpfer, et al., 1995). For example, oils with a high level of C12:0 can be used in manufacturing of detergent, shampoo, margarine and shortening. Soybean oil with high levels of C16:0 (23-26%) have a greater oxidative stability than conventional soybean oil (11% C16:0), thus reducing the need for hydrogenation in producing some food products, such as shortening and margarine (Shen et al., 1997).

Some non-crop plant species produce seed oils with high percentages of MCFA, partly due to the function of their special acyl-acyl carrier protein (ACP) thioesterases (TE). TE hydrolyses the thioester bond between the elongating fatty acyl group and the co-factor ACP in the biosynthetic process, thus prematurely terminating the elongation process and leading to the accumulation of medium-chain fatty acids (MCFA) in the seed oil (Jones et al., 1995; Ohlrogge et al., 1978; Töpfer et al., 1995). However, the levels of the fatty acids targeted by TE in *B. napus* transgenic lines are usually lower than in the original source of the TE (Dehesh et al., 1996; Hawkins and Kridl, 1998; Jones et al., 1995; Voelker et al., 1997). In seed oil of California bay tree (*Umbellularia californica*) the level of C12:0 and C10:0 can be up to 70% (Pollard et al., 1991; Töpfer, 1995). Plants

transformed with the TE gene (*Uc FatB1*) cloned from bay tree had less than 60% C12:0 (Voelker et al., 1996). *Cuphea hookeriana* seed oil contains up to 50% C8:0 and 25% C10:0 (Dehesh et al., 1996). Transformation with a TE gene (*Ch FatB2*) from cuphea plants produced plants with less than 40% C8:0 and C10:0. Similarly, reduced levels of the target fatty acids were found in transformed *B napus* plants carrying TE genes isolated from elm, nutmeg (Voleker et al., 1997), and mangosteen (*Garcinia mangostana*) (Hawkins and Kridl, 1998).

Several explanations for the lower levels of the TE targeted fatty acids in transgenic *B. napus* background have been proposed. These included differential activity and substrate specificity of the enzymes involved in the fatty acid biosynthetic pathways in the *B. napus* genetic background, e.g. β-ketoacyl ACP synthase (KAS) which catalyzes the elongation of fatty acyl chain-ACP and acyl-ACP acyltransferase which assembles fatty acids into triacylglycerols (Dehesh et al., 1996; Hawkins and Kridl, 1998; Knutzon et al., 1992; Voelker et al., 1997).

The effect of recipient genotype on the expression of transgenes has been reported in a number of plant species (Blundy et al., 1991; Scott et al., 1998; Xu et al., 1997). For example, a study with a white clover line (*Trifolium repens* L.) carrying an intact copy of the GUS gene showed a four-fold variation in the GUS activity between plants, even though all of the plants contained one copy of the same T-DNA insert (Scott et al., 1998). Progeny from plants with a high level of expression showed a higher GUS activity in the next generation than those from plants with a low expression, suggesting an influence from genetic factors in the recipient genotypes.

Influence of genotypic background on the fatty acid composition of seed oils has also been observed in studies with non-transgenic oilseed plants. In some studies with crosses between plants having contrasting levels of fatty acids, it was observed that the fatty acid composition was controlled completely by the embryonic genotype of the seed. The fatty acid levels were not affected by the genotype of the maternal plant, such as the C22:1 level in *B. napus* (Downey and Harvey, 1963) and the C16:0 level in soybean (*Glycine max*) (Kinoshita et al., 1998; Narvel et al., 2000). In other cases, influence from the maternal plant was detected although the embryonic genotype was the

determining factor in the level of the fatty acids, such as the C18 fatty acids (C18:1, C18:2 and C18:3) in *B. napus* (Pleines and Friedt, 1989; Thomas and Kondra, 1973; Rakow, 1973), the C18:0 level in sunflower (*Helianthus annuus* L.) (Perez-Vick et al., 1999), and the levels of C18 fatty acids in flax (*Linum usitatissimum*) (Yermanos and Knowles, 1962). Crossing experiments also detected cytoplasmic effects on fatty acid level, as shown by significant differences between F_1 populations from reciprocal crosses in *B.napus*, maize (*Zea mays* L.) and soybean (Brim et al., 1968; de la Roche et al., 1971; Diepenbrock and Wilson, 1987; Poneleit and Bauman, 1970).

In this study, four transgenic parental lines transformed with different TE transgenes were crossed with three non-transgenic genotypes. Comparative analyses were conducted between F_1 seeds and between DH lines originating from different crosses in order to determine: i) the effect of recipient genotype, ii) maternal effect, and iii) cytoplasmic effect, on the production of the fatty acids targeted by TE transgenes. In addition, the interaction of different TE transgenes was assessed with F_1 seeds from crosses between transgenic lines carrying different TE.

3.3 MATERIALS AND METHODS

3.3.1 Parental Genotypes

Nine *B. napus* genotypes, including four transgenic parental lines, two non-transgenic breeding lines used for transformation of the four transgenic lines and three non-transgenic cultivars with distinct fatty acid compositions, were used as parents in this study (Table 3.1). The original seeds of the four transgenic lines, as well as the two breeding lines '212/86' and 'QO4', were kindly provided by Calgene Inc. (USA). Transgenic line TL1 is transformed with the bay-TE gene *Ua FatB1* (Voelker et al., 1992, 1996), TL3 with the elm-TE *Uc FatB1*, TL5 with the nutmeg-TE *Mf FatB1* (Voelker, 1997), and TL6 with the cuphea-TE gene *Ch FatB1*(Jones et al., 1995). Genotype 212/86, a late-maturing Danish breeding line with <1% C22:1, was transformed for the development of TL1, TL3 and TL6; QO4, a selection from a low linolenic acid cultivar Stellar, was transformed for TL5

Table 3.1 Fatty acid composition of the nine genotypes used as the parents in this study.

Genotype	No. of plants	Fatty acid									
		C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:1	C22:1
		%									
Transgenic parent:											
TL1	9	-†	45.2(1.5)‡	4.6(0.2)	3.1(0.1)	1.3(0.1)	25.5(1.3)	11.0(0.4)	7.9(0.3)	-	-
TL3	12	2.4(0.5)	1.3(0.1)	12.0(0.6)	31.6(1.1)	3.1(0.2)	26.5(1.4)	13.5(0.5)	7.3(0.4)	-	-
TL5	12	-	-	14.0(1.0)	27.7(0.9)	5.9(0.1)	32.3(1.1)	15.8(0.8)	1.0(0.1)	-	-
TL6	14	-	-	1.8(0.2)	35.7(1.5)	2.1(0.1)	33.5(1.2)	15.6(0.5)	9.1(0.3)	-	-
Non-transgenic parent:											
Apollo	12	-	-	-	4.4(0.1)	2.2(0.2)	69.5(1.4)	19.2(1.3)	1.7(0.2)	1.2(0.0)	-
AC Excel	13	-	-	-	3.9(0.3)	1.4(0.2)	67.8(0.8)	17.1(0.6)	7.6(0.4)	1.2(0.1)	-
Mercury	14	-	-	-	3.0(0.1)	0.8(0.0)	21.2(1.3)	10.7(0.3)	6.5(0.6)	8.8(0.6)	45.5(1.2)
212/86	7	-	-	-	5.1(0.3)	1.6(0.1)	65.5(1.0)	18.7(0.6)	5.8(0.6)	1.9(0.2)	-
QO4	7	-	-	-	4.0(0.6)	2.3(0.1)	68.9(1.7)	18.0(1.5)	1.5(0.1)	1.7(0.2)	-

† Less than 1%.
‡ Standard deviation in parenthesis.

development (M. Sovero, personal comm.). Of the three non-transgenic cultivars, AC Excel is a canola quality cultivar with less than 2% C22:1 in the fatty acid composition of the seed oil (Rakow, 1993). Apollo has a fatty acid composition typical of low C18:3 canola cultivars with less than 2% C22:1 and less than 3% C18:3 (Scarth et al., 1995a). Mercury is a high erucic acid rapeseed (HEAR) cultivar with over 50% C22:1 in the seed oil (Scarth et al., 1995b).

3.3.2 Production of F_1 Seeds

Two crossing experiments were conducted with the nine parental genotypes for the production of the F_1 seeds used in this study. In the first experiment, 24 crosses were made by reciprocally crossing each of the four transgenic lines with each of the three cultivars. The parental plants were grown in a controlled environment with a 16-h photoperiod, 580 $\mu E\ m^{-2}\ s^{-1}$ light intensity, and temperatures of 20/15 °C at day/night. Four racemes of each transgenic parental plant were used, with three racemes being pollinated separately with the three cultivars and the remaining raceme being self-pollinated. Reciprocal crossing was conducted by cross-pollinating individual plants of the two parents in pair.

In the second experiment, the transgenic parental plants were grown from self-pollinated seeds from the first experiment. Sixteen crosses were made by crossing the four transgenic lines with each other, and also with either QO4 or 212/86, the recipient genotype used in the transformation with the TE transgenes. The transgenic parental plants were also bagged at flowering to produce self-pollinated seeds.

For the both experiments, F_1 seeds and self-pollinated seeds were harvested at maturity from the individual parental plants and the fatty acid composition was determined as described below.

3.3.3 DH Line Development

DH lines were developed from 18 of the 24 crosses made in the first crossing experiment, which included 12 crosses from reciprocally crossing TL1 and TL6 with the three cultivars, i.e. (TL1 or TL6) / (Apollo, AC Excel or Mercury), and 6 crosses from crossing TL3 and TL5 as the female with

the three cultivars as the male parents, i.e. (TL3 or TL5) x (Apollo, AC Excel or Mercury). The DH line development procedure was basically as described by Ferrie and Keller (1995). Immature pollen grains were isolated from unopened flower buds on F_1 hybrid plants. DH_1 plants were grown in a glasshouse and self-pollinated to produce DH_2 seeds which were tested for the fatty acid composition.

3.3.4 Determination of Fatty Acid Composition

The fatty acid composition of seed oil was determined by gas chromatography of the methyl ester derivatives of the fatty acids (Hougen and Bodo, 1973; Knutzon et al., 1992). A sample of 10 seeds was picked randomly from the seeds of each plant to be tested; the oil was extracted overnight with 1 ml of heptane. 300 ul of 0.5 M sodium methoxide was added for methyl ester derivitization. The oven temperature was programmed to increase from 190 to 230 °C. The level of each fatty acid is reported as the percentage of the total fatty acids.

3.3.5 Identification of Transgenic DH Lines

Transgenic DH lines were identified mainly based on the level of the target fatty acids in DH_2 seeds. As well, DH lines with a relatively lower level of the target fatty acids were further tested for the TE transgenes with the PCR procedure as described by Floolad et al. (1994). Plant genomic DNA was extracted from cotyledon and young leaves with the CTAB procedure reported by Kidwell and Osborn (1992). Extracted DNA was purified with 25:24:1 of chloroform, phenol and isoamyl alcohol (Ausubel et al., 1995). The sequences of the left and right primers for the amplification of an internal fragment of each TE transgene and a control band are presented in Table 3.2. The primers for the control band were designed based on a napin gene sequence published by Kridl et al. (1991), and were included in each PCR reaction. Both transgenic and non-transgenic plants showed this band in PCR tests.

3.3.6 Statistical Analysis

Statistical calculations and multiple comparisons were performed as described by Ott (1993).

Table 3.2 Sequence of the primers used to identify the transgenic DH lines for each of the four TE transgenes by PCR analysis and the expected size of the PCR products.

Transgene/ control	Sequence from 5' to 3'		Size of expected PCR product
	Left primer	Right primer	kb
Bay-TE	GAGCTTGAAAAGGTTGCCTG	GGTTCTGCGGGTATCACACT	0.98
Elm-TE	TCCACAACAGCACCATCATT	CTTGCTGCAATCACGACTGT	0.95
Nutmeg-TE	CTCTCCGTCTCCAGTGAAGC	CAAACCGTCCTTTCCACACT	1.05
Cuphea-TE	GAACTTTTATCAACCA	ACCTGCCCTTCACTCAG	1.07
Control†	TGGATTATGGAACAATGGGAA	CCACCATCTTACGCTTTGGTA	0.54

† Designed based on the sequence of a napin promoter gene published by Kridl et al. (1991) to amplify a band for either transgenic and non-transgenic plants.

3.4 RESULTS

3.4.1 Identification of Transgenic DH Lines

A total of 1334 DH lines were developed from the TL1-, TL3-, TL5- and TL6-crosses, i.e. the crosses between the transgenic parental lines and the three cultivars (Table 3.3). Since the microspore donor plants were hybrids for the TE transgenes, it was expected that the DH lines would segregate for the target fatty acids, with some lines being transgenic and accumulating C12:0 (the target fatty acid of the bay-TE) or enhanced level of C16:0 (the target fatty acid of the other three TE), and the others being non-transgenic and having a fatty acid composition similar to that of non-transgenic plants. Transgenic DH lines could be identified based on the level of the target fatty acids. However, there was a wide variation, with some of the lines having lower levels of the target fatty acid than others. For transgenic lines with lower levels of the target fatty acids, the presence of the TE transgene was confirmed by PCR tests.

Table 3.3 Number of lines and minimum and maximum level of the target fatty acids for the DH lines developed from crosses of the four transgenic parents TL1, TL3, TL5 and TL6 with the three cultivars, Apollo (A), AC Excel (E) and Mercury (M).

Cross	Transgene	Target fatty acid	Total no. of DH lines	Transgenic line			Non-transgenic line		
					Target fatty acid			Target fatty acid	
				No.	min.	max.	No.	min.	max.
			no.	no.	%	%	no.	%	%
TL1/(A, E or M)	Bay-TE	C12:0	232	113	6.9	48.2	119	-†	-
TL3/(A, E, or M)	Elm-TE	C16:0	94	70	8.3	38.4	24	4.6	5.9
TL5/(A, E, or M)	Nutmeg-TE	C16:0	275	143	15.7	32.4	132	3.2	6.9
TL6/(A, E, or M)	Cuphea-TE	C16:0	733	636	12.4	43.8	97	2.7	8.5
Total:			1334	962			372		

† Less than 1%.

Bay-TE Transgenic Lines

Identification of the bay-TE transgenic lines was based on the C12:0 level of the seed oils and PCR tests for the bay-TE transgene. As shown by the distribution of the C12:0 level (Fig. 3.1), the DH lines from the TL1-crosses could be separated into two groups. One group of 119 lines had a C12:0 level of less than 1% in the seed oil, and the second group of 113 lines had a minimum C12:0 level of 6.9% (Table 3.3). Because conventional canola cultivars do not accumulate C12:0 in the seed oil, the first group of lines were considered non-transgenic and the second group transgenic. In the second group, transgenic lines with relatively lower levels of C22:1(6.9-20.4%) than the others were further confirmed by PCR tests for the bay-TE transgene (Fig. 3.2). The PCR results showed that DH lines with 6.9% or higher levels of C12:0 did have the TE gene as represented by a band of ca. 1.0 kb that was the expected size for the PCR product for the bay TE transgene, whereas the lines with less than 1% C12:0 showed only the control band as the non-transgenic control plants.

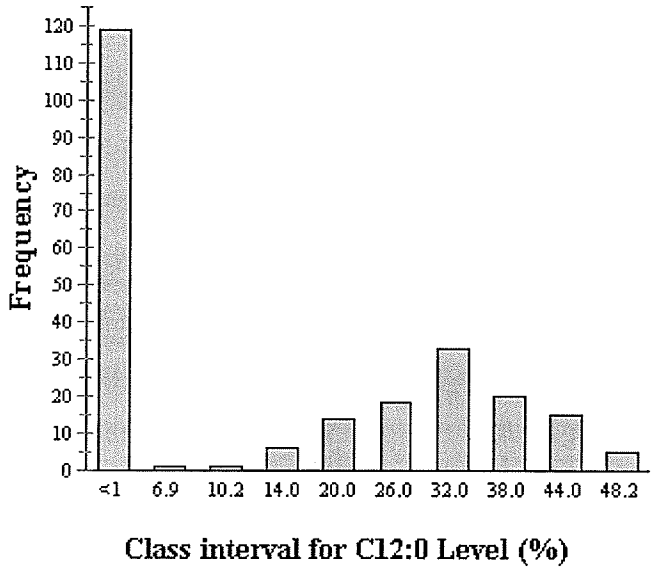
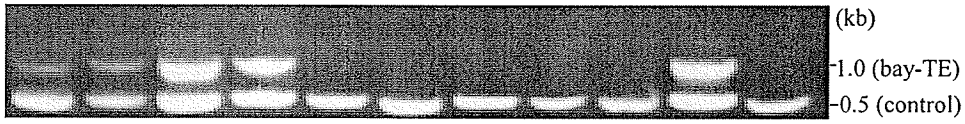


Figure 3.1 Frequency distribution of the C12:0 level (%) of DH lines developed from crosses between the bay-TE transgenic parental line TL1 and the three non-transgenic cultivars Apollo, AC Excel and Mercury.



	1	2	3	4	5	6	7	8	9	10	11
C12:0(%):	20.4	17.1	15.6	6.9	<1	<1	<1	<1	<1	40.0	<1

Figure 3.2 PCR analysis of DH lines with TL1 as the transgenic parent. In each PCR reaction the primers for amplifying both the bay-TE transgene and the napin promoter sequence were added. Lane 1-9, DH lines; lane 10, positive control (the transgenic parent TL1); Lane 11, negative control (the non-transgenic genotype 212/86). The 12:0 levels of the DH lines are presented under the lane no. The sizes of the bands were labeled in kilobase (kb).

Elm-TE Transgenic Lines

Identification of elm-TE transgenic DH lines developed from TL3-crosses was based on the C16:0 level and PCR tests for the elm-TE transgene. The distribution of the target fatty acid C16:0 showed that the DH lines from TL3-crosses could be divided into two groups, similar to the DH lines from TL1-crosses (Fig. 3.3), with one group of lines each producing less than 6% C16:0 and the second group having a minimum C16:0 level of 8.3% (Table 3.3). In the second group, only two plants had slightly more than 8% C16:0 and all the others had more than 15% C16:0 (Fig. 3.3). PCR tests for the elm-TE transgene confirmed that the DH plants with more than 8% C16:0 had the elm-TE as represented by the band of the expected size, those with less than 6% did not (Fig. 3.4).

Nutmeg-TE and Cuphea-TE Transgenic Lines

DH lines carrying the nutmeg- or cuphea-TE transgenes were identified by analyses of the target fatty acid C16:0 and PCR testes. Of the DH lines from TL5-crosses and TL6-crosses, 143 and 636 plants were transgenic for the nutmeg-TE and the cuphea-TE transgenes, respectively (Table 3.3).

3.4.2 Effect of Recipient Genotypes on the Specificity of the TE for the Target Fatty Acids

Although C12:0 and C16:0 were the major fatty acids targeted by the bay-TE and the other three TE, respectively, these TE also showed activity on other acyl-ACP substrates, thus leading to accumulation of more than one target fatty acids; e.g., bay-TE transgenic lines also accumulated myristic acid (C14:0) in addition to C12:0 in the seed oil. Examination of transgenic DH lines from crosses with different non-transgenic parents showed that the relative specificity of the TE for the

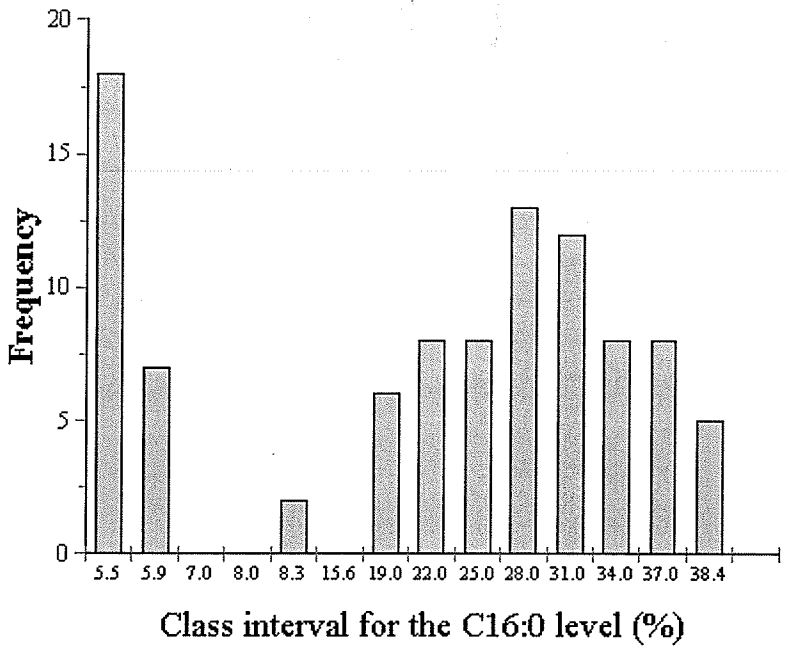


Figure 3.3 Frequency distribution of the C16:0 Level (%) for the DH lines developed from crosses between the elm-TE transgenic parental line TL3 and the three non-transgenic cultivars Apollo, AC Excel and Mercury.

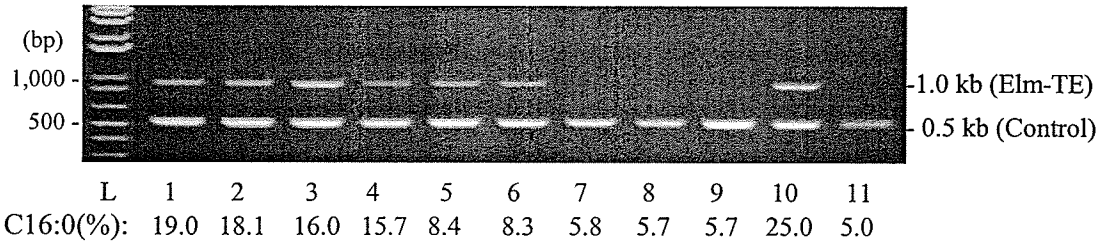


Figure 3.4 PCR analysis of DH lines developed from the crosses with TL3 as the transgenic parent. In each PCR test primers for amplification of an 1.0-kb fragment of the elm-TE and a 0.5-kb fragment of the napin promoter sequence were added. L, DNA molecular weight marker labeled in base pairs (bp); lanes 1-9, DH lines; lane 10, transgenic parent; lane 11, non-transgenic cultivar 212/86.

target fatty acids were similar when the TE being expressed in different recipient genotypes as described below.

The bay-TE transgenic lines were divided into four groups based on the non-transgenic parent and the C22:1 level of the lines in order to determine the effect of recipient genotype on the specificity of the bay-TE for the target fatty acids (Table 3.4). The DH lines from the crosses of Mercury and the transgenic parents segregated for the C22:1 level, while the lines from the crosses of the two low C22:1 parents (Apollo and AC Excel) and the transgenic parent TL1 did not segregate. The mean level of the target fatty acid of the lines in the same groups was displayed for each group in Fig. 3.5a. Regardless of the differences in the non-transgenic parental genotype and the C22:1 level, all of the four groups accumulated C12:0 and C14:0, the two target fatty acids of the bay-TE, in the seed oils. In addition, all of the four groups showed much higher C12:0 than C14:0, implying that the relative specificity in the target fatty acids of the bay-TE was similar in different recipient genotypes, with C12:0 being the major target fatty acid.

Table 3.4 Number of transgenic DH lines developed from each cross of the four transgenic parents TL1, TL3, TL5 and TL6 with Apollo, AC Excel or Mercury.

		Non-transgenic parent			
Transgenic				Mercury†	
parent	Transgene	Apollo	AC Excel	Low C22:1	High C22:1
TL1	Bay-TE	43	49	4(<1)‡	16(24.4)
TL3	Elm-TE	15	51	-§	4(14.5)
TL5	Nutmeg-TE	48	60	25(<1)	10(17.0)
TL6	Cuphea-TE	275	201	68(<1)	92(22.9)

† DH lines from Mercury as the non-transgenic parent were grouped based on the C22:1 level of the seed oil.
‡ The mean C22:1 level was in parenthesis.
§ Not available.

The transgenic DH lines carrying the elm-TE, nutmeg-TE or cuphea-TE transgenes produced only traces of C12:0, but much higher levels of C16:0 than the non-transgenic genotype (ca. 4% C16:0)

a) Bay-TE DH lines:

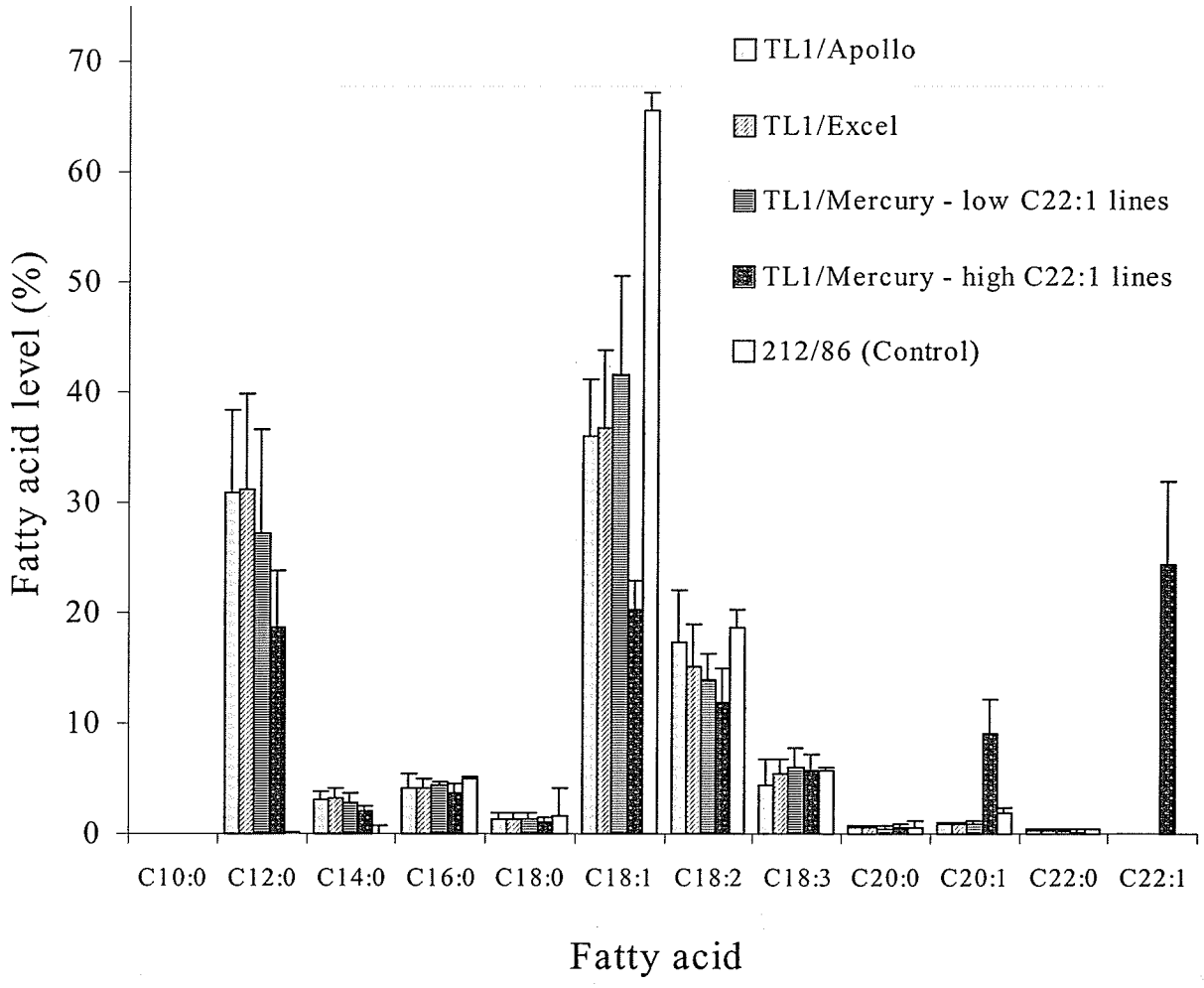


Figure 3.5 Fatty acid compositions of transgenic DH lines with different non-transgenic parents and different C22:1 levels. The fatty acid profiles of non-transgenic genotypes 212/86 and QO4 were included as the controls. Each of the four graphs shows the mean fatty acid composition of the DH lines developed from each hybrid between a transgenic parental line and the three cultivars (Apollo, AC Excel and Mercury), with the DH lines from Mercury as the non-transgenic parent being divided into two groups: lines with a low C22:1 level (<1) and lines with a high C22:1 level (ca. 20% on average, see the graph). a) The DH lines from the bay-TE transgenic parental line TL1. b) The DH lines from elm-TE parental line TL3. c) The DH lines from nutmeg-TE parental line TL5. d) The DH lines from the cuphea-TE parental line TL6. Error bars represent the standard deviation.

Figure 3.5 continued:

b) Elm-TE DH lines:

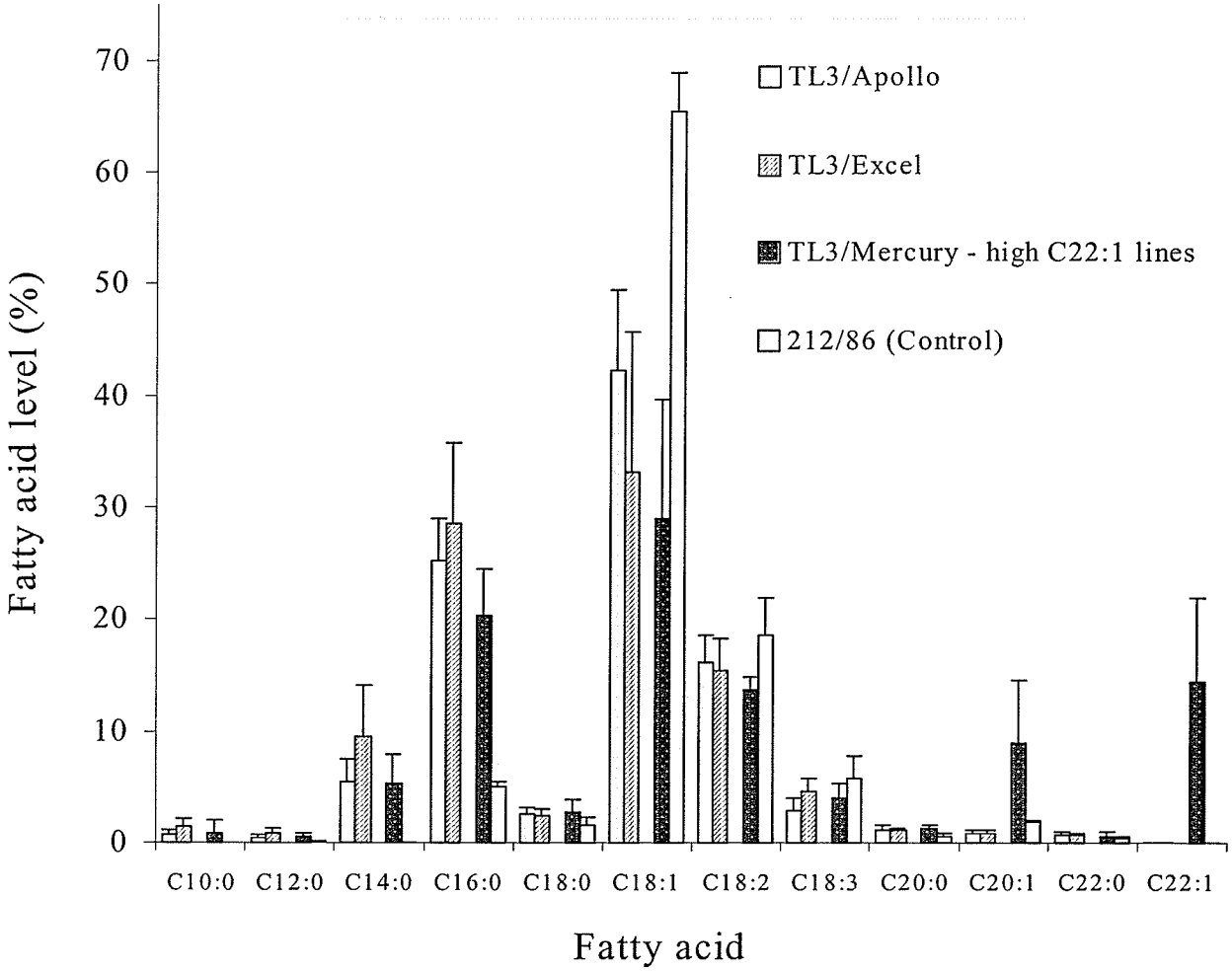


Figure 3.5 continued:

c) Nutmeg-TE DH lines:

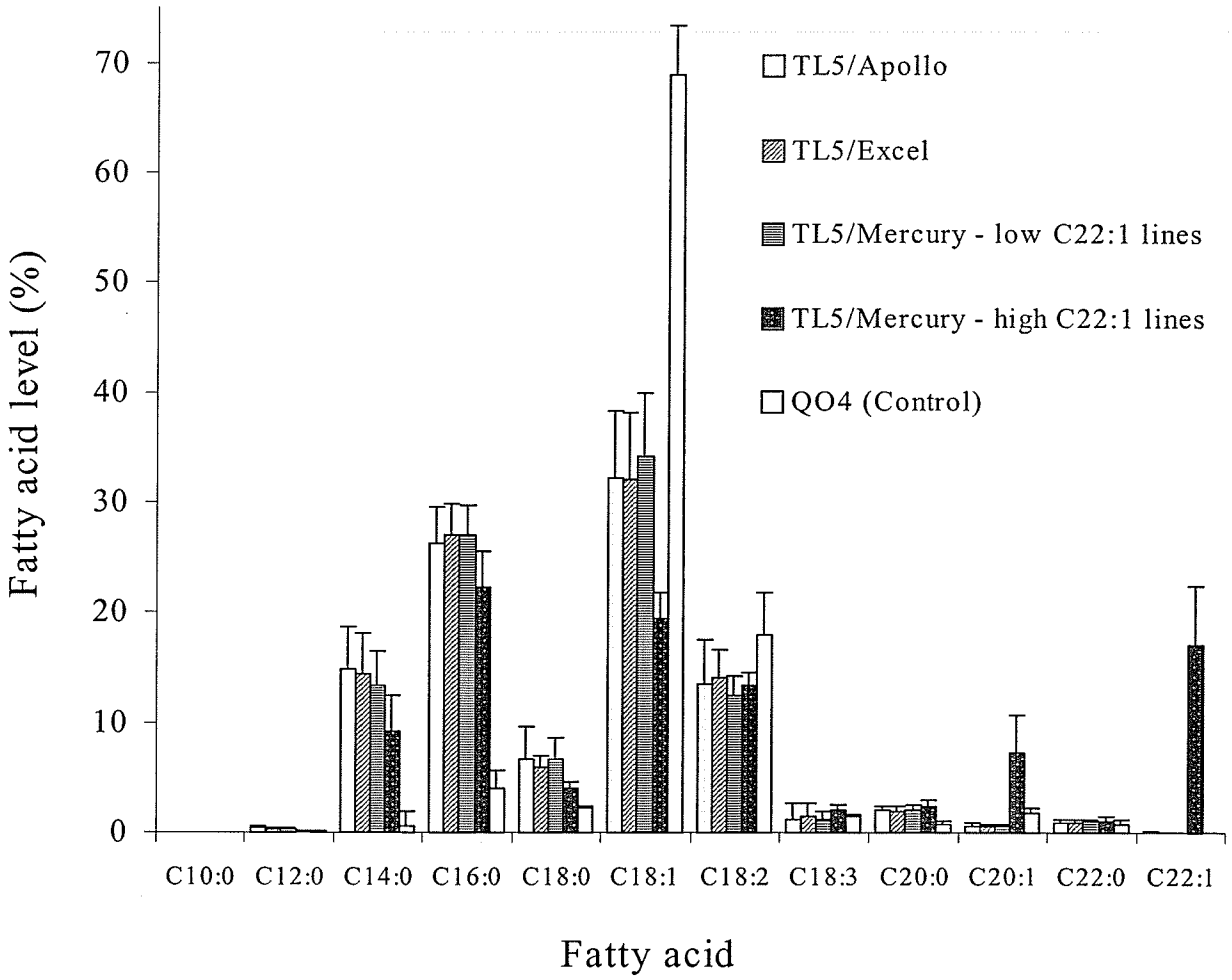
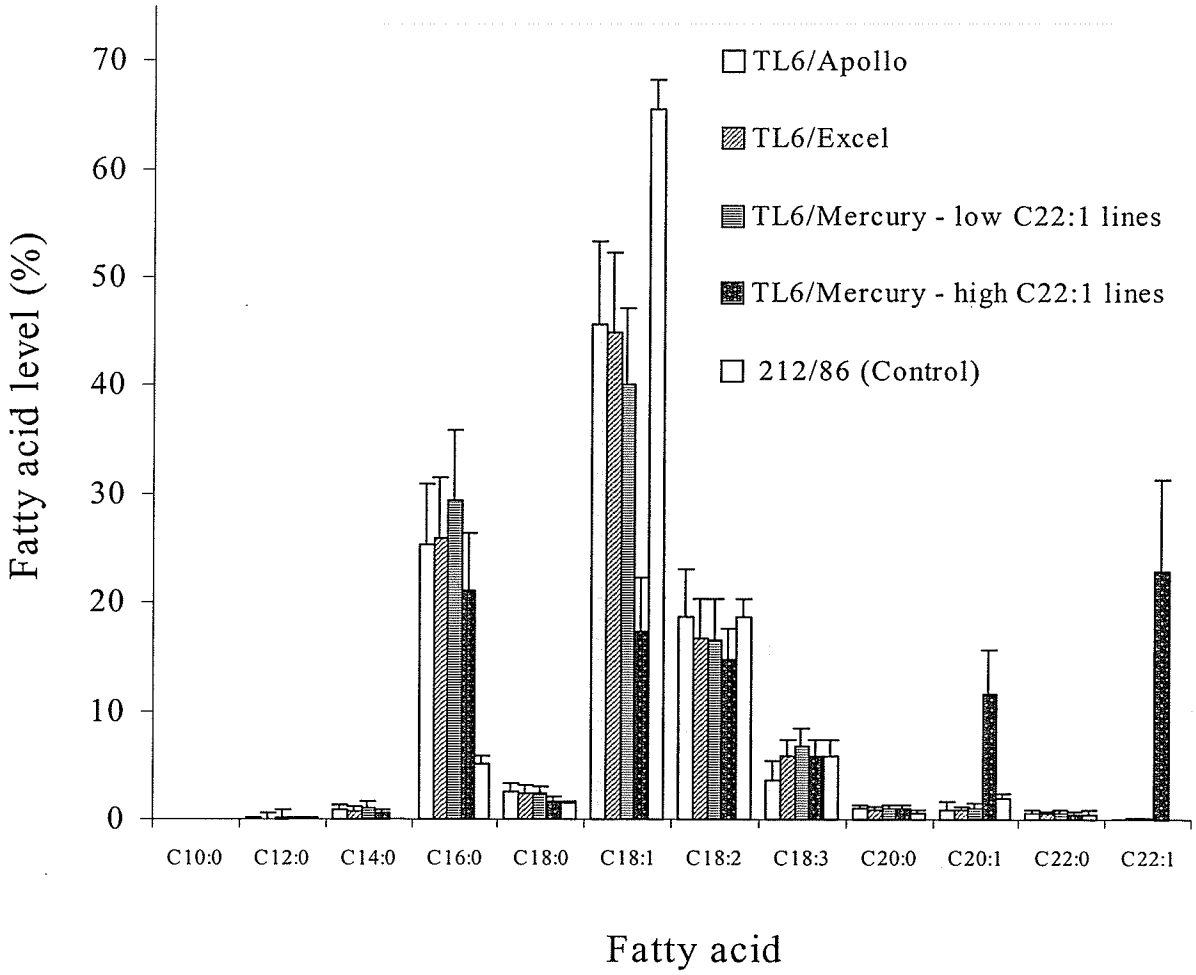


Figure 3.5 continued:

d) Cuphea-TE DH lines:



(Fig. 3.5b-d). Elm-TE and nutmeg-TE transgenic lines also accumulated C14:0 in the seed oil (Fig. 3.5b, c); however, the C14:0 level was always much lower than the level of the major target fatty acid C16:0. For the cuphea-TE lines, C16:0 was also the major target fatty acid, with only traces of C14:0 (<1%, Fig. 3.5d). Therefore, the relative specificity of the TE for the target fatty acids was similar in different recipient genotypes carrying the same TE.

3.4.3 Effect of Recipient Genotype on the Level of the Target Fatty Acids in F_1 seeds

F_1 seeds from crosses of the same transgenic female parent with different non-transgenic male parents have a common genotype of the maternal plants, as well as the same cytoplasm. The genotype of the embryo in the seeds is distinct for each cross, since half number of the chromosomes of the seeds are inherited from the non-transgenic parent. Thus, a significant difference in the level of the target fatty acid between F_1 seeds from different crosses would be an indication for the effect of recipient genotypes on the target fatty acid.

Influence of recipient genotype on the level of the target fatty acids was detected by comparing F_1 seed from crosses with Apollo and Mercury as the non-transgenic parents (Table 3.5). The mean level of C12:0, targeted by the bay-TE, in TL1 x Apollo seeds (37.3%), was significantly higher than that in TL1 x Mercury seeds (26.2%). As well, transgenic seeds expressing the elm-TE, nutmeg-TE or cuphea-TE transgene from crosses TL3 x Apollo, TL5 x Apollo and TL6 x Apollo, respectively, showed significantly higher levels of the target fatty acid C16:0 than seeds which expressed the same TE but from crosses with Mercury as the non-transgenic parent. Unlike F_1 seeds with Mercury as the non-transgenic parent, F_1 seeds from Apollo produced only traces of C22:1, less than 1% (Table 3.5). Thus, the differences in the levels of the target fatty acids between the F_1 seeds could be associated with C22:1 accumulation; alternatively, it could be related to other genetic differences between the non-transgenic parents Apollo and Mercury.

Comparison between F_1 seeds from AC Excel and Mercury as the non-transgenic parents also showed the effect of recipient genotype (Table 3.5). With AC Excel as the non-transgenic parent, the F_1 seeds with either the bay-TE, elm-TE or cuphea-TE transgenes had significantly higher levels

of the target fatty acids than the F_1 seeds with the same transgene but from Mercury as the non-transgenic parent.

Table 3.5 Mean fatty acid level of F_1 seeds from each cross of the four transgenic parents TL1, TL3, TL5 or TL6 as the female with Apollo, AC Excel or Mercury as the male parents.

Transgenic parent (TE)	Fatty acid	Non-transgenic parent			No. of replicates
		Apollo	AC Excel	Mercury	
		%			no.
TL1 (bay-TE)	C12:0	37.3 a†	35.5 a	26.1 b	3
	C18:3	5.5 b	8.0 a	8.1 a	
	C22:1	-‡	-	19.9	
TL3 (elm-TE)	C16:0	31.2 a	31.8 a	26.9 b	10
	C18:3	4.1 c	6.4 b	7.0 a	
	C22:1	-	-	14.3	
TL5 (nutemg-TE)	C16:0	25.1 a	24.4 ab	22.8 b	6
	C18:3	1.1 a	1.7 a	1.2 a	
	C22:1	-	-	12.6	
TL6(cuphea-TE)	C16:0	29.6 a	29.9 a	27.5 b	10
	C18:3	4.9 b	8.1 a	7.9 a	
	C22:1	-	-	18.3	

† The means in the same row followed by the same letter are not significantly different at the 0.05 level.

‡ Less than 1%.

However, Apollo as the non-transgenic parent was not significantly different from AC Excel as the non-transgenic parent in the level of the target fatty acids for all the four TE transgenes (Table 3.5). For example, TL3 x Apollo seeds and TL3 x AC Excel seeds had similar C16:0 levels, 31.2% and 31.8%, respectively, although the F_1 seeds had significantly different C18:3 levels. The results indicated that the seed embryo genotypes, in which half of the chromosomes were from Apollo or AC Excel and the other half from the same transgenic parent, were not significantly different in their influence on the accumulation of the target fatty acids.

3.4.4 Effect of Recipient Genotype on the Level of the Target Fatty Acids in DH lines

DH lines with Different C18:3 Levels

Although comparative analyses in the section above with F_1 seeds demonstrated the effect of recipient genotype on the target fatty acids, it could not be determined whether the effect was associated with the levels of C18:3 and C22:1 as discussed above.

Effect of recipient genotype with different C18:3 levels on the accumulation of the target fatty acids was tested with DH lines from six crosses (Table 3.6). In the six crosses, the transgenic parents TL1, TL3 and TL5 were high and Apollo was low in the C18:3 level. DH lines with contrasting C18:3 levels were selected from the same crosses and compared to determine the influence of the accumulation of C18:3 on the target fatty acid level.

Table 3.6 Mean, minimum and maximum level of linolenic acid (C18:3) and the target fatty acids for transgenic DH lines carrying the bay-TE, the elm-TE or the cuphea-TE transgene.

			Fatty acid level					
Trans-		No. of	C18:3			Target fatty acid†		
gene	Cross‡	DH lines	mean	min.	max.	mean	min.	max.
		no.	%					
Bay-TE	TL1/Apollo	43	4.38	1.5	12.9	30.9	10.2	43.8
Elm-TE	TL3xApollo	14	2.95	0.9	5.7	25.2	18.1	32.9
Cuphea-TE	TL6/Apollo	275	3.62	0.9	12.6	25.3	12.8	43.8

† The target fatty acid was C12:0 for the bay-TE, C16:0 for the elm-TE and cuphea-TE.
‡ The crosses from which the DH lines were developed. TL1/Apollo represents the reciprocals TL1 x Apollo and Apollo x TL1, TL6/Apollo for TL6 x Apollo and Apollo x TL6.

Based on the bay-TE transgenic DH lines with contrasting C18:3 levels from TL1/Apollo, the accumulation of C18:3 did not significantly influence the level of C12:0 targeted by the bay-TE. The bay-TE lines from TL1/Apollo displayed a continuous distribution in the C18:3 level, which ranged from 1.5% to 12.9% with an average of 4.38% (Table 3.6). As well, the C12:0 level showed continuous variation over the range of 10.2 to 43.8%. Correlation analysis of the two variables

(C18:3 level and C12:0 level) of these lines showed that the levels of the two fatty acids were not correlated (r = 0.03), indicating that the level of the target fatty acid C12:0 was not significantly influenced by the accumulation of C18:3. The average C18:3 level (4.38%) was used as division between high and low C18:3 lines for the DH lines from TL1/Apollo. The high C18:3 lines had a mean C18:3 level of 6.5%, and the low C18:3 lines had a mean C18:3 level of 2.7% (Table 3.7). Although the two groups had contrasting C18:3 levels, the average C12:0 levels were almost identical.

Table 3.7 Mean level of linolenic acid (C18:3) and the target fatty acids for transgenic DH lines with high or low C18:3 levels carrying the bay-TE, elm-TE or cuphea-TE transgenes.

Transgene	Group of DH line	No. of lines	Fatty acid level	
			C18:3	Target fatty acid†
		no.	%	
Bay-TE	High C18:3 lines	19	6.5	29.5a
	Low C18:3 lines	24	2.7	32.1a
Elm-TE	High C18:3 lines	5	4.0	26.8a
	Low C18:3 lines	9	2.4	24.3a
Cuphea-TE	High C18:3 lines	119	5.2	25.4a
	Low C18:3 lines	156	2.4	25.3a

† The target fatty acid was C12:0 for the bay-TE lines and C16:0 for elm- and cuphea-TE lines. The means followed the same letter for the lines carrying the same TE transgene were not significantly different at the 0.05 level.

Elm-TE transgenic DH lines from TL3/Apollo and cuphea-TE transgenic lines from TL6/Apollo also varied in the C18:3 level (Table 3.6). Similar to the observation in the bay-TE lines, the level of the target fatty acid C16:0 in the elm- and cuphea-TE lines was not correlated with the C18:3 level (r = 0.40 and 0.03, respectively). High C18:3 lines expressing the elm- or the cuphea-TE accumulated similar levels of C16:0 in the seed oil as low C18:3 lines expressing the same TE (Table 3.7). Thus, it can be concluded that the difference in the C18:3 level between recipient genotypes did not influence the level of C16:0 targeted by the elm-TE and cuphea-TE transgenes.

DH Lines with Different C22:1 Levels

The effect of recipient genotype with different C22:1 levels was determined by analysis of DH lines from crosses of the transgenic parental lines and the high C22:1 cultivar Mercury. As expected, no segregation in the C22:1 level was observed among DH lines from crosses with Apollo and AC Excel as the non-transgenic parents since both the cultivars and the transgenic parents had less than 1% C22:1 (Table 3.1).

Transgenic DH lines from crosses with Mercury as the non-transgenic parent segregated for the C22:1 level, and the lines with contrasting C22:1 levels showed significantly different levels of the target fatty acids (Table 3.8). The four bay-TE DH lines with low C22:1 (<1%) from TL1/Mercury

Table 3.8 Mean level (%) of the target fatty acids and erucic acid (C22:1) for transgenic DH lines developed from each cross of the transgenic parents TL1, TL3, TL5 and TL6 with Apollo, AC Excel or Mercury.

Transgenic parent	Parameter	Non-transgenic parent			
				Mercury†	
		Apollo	AC Excel	Low C22:1	High C22:1
Bay-TE TL1	C12:0 (%)	30.9 a‡	31.1 a	27.1 a	18.7 b
	No. of lines	43	49	4	16
	C22:1(%)	<1	<1	<1	24.4
Elm-TE TL3	C16:0(%)	25.2 ab	28.6 a	N.A. §	20.3 b
	No. of lines	15	51	N.A.	4
	C22:1(%)	<1	<1	N.A.	14.5
Nutmeg-TE TL5	C16:0(%)	26.4 a	27.0 a	27.0 a	22.2 b
	No. of lines	48	60	25	10
	C22:1(%)	<1	<1	<1	17.0
Cuphea-TE TL6	C16:0(%)	25.3 b	25.9 b	29.4 a	21.1 c
	No. of lines	275	201	68	92
	C22:1(%)	<1	<1	<1	22.9

† DH lines from crosses with Mercury as the non-transgenic parent were divided into lines with low or high C22:1 levels; with the average C22:1 level for each group being presented.

‡ The means with different letters in the same rows are significantly different at the 0.05 level.

§ Not available.

had a mean C12:0 level of 27.1%, which is significantly higher than the average C12:0 level of the high C22:1 lines from the same cross with an average C22:1 of 24.4%. Nutmeg- and cuphea-TE transgenic lines with low C22:1 levels also produced significantly higher levels of the target fatty acid C16:0 (17% and 23%, respectively) than the low C22:1 lines carrying the same transgenes. These results indicated that, as observed with F_1 seeds (Section 3.4.2), the expression of the TE transgenes in low C22:1 recipient genotypes increased the level of the target fatty acids compared to the expression in high C22:1 genotypes.

Although the low C22:1 DH lines showed higher levels of the target fatty acids than the high C22:1 DH lines, the differences in the target fatty acid level between the low and the high C22:1 lines were lower than expected due to the reduced percentage of C22:1 in the low C22:1 genotypes (Table 3.9).

Table 3.9 Fatty acid composition of DH lines with high or low levels of erucic acid (C22:1) carrying the bay-TE, nutmeg-TE or cuphea-TE transgenes developed from crosses of transgenic parental lines TL1, TL5 and TL6 with the high C22:1 cultivar Mercury.

Transgenic parent (TE)	No. of lines	Fatty acid level										Avg. Ratio†
		C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:1	
		%										
TL1 (bay-TE)												
High C22:1 lines	16	18.7	2.1	3.6	1.0	20.4	12.0	5.7	0.6	9.2	24.4	-‡
Low C22:1 lines	4	27.1	2.8	4.4	1.0	41.7	14.0	6.0	0.5	0.9	0.0	-
Ratio§		1.45	1.32	1.21	1.03	2.05	1.17	1.06	0.70	0.10	-	1.51
TL5 (nutmeg-TE)												
High C22:1 lines	10	-	9.2	22.2	4.0	19.5	13.4	2.0	2.4	7.3	17.0	-
Low C22:1 lines	25	-	13.4	27.0	6.7	34.1	12.5	1.2	2.1	0.6	0.0	-
Ratio		-	1.45	1.22	1.68	1.75	0.93	0.60	0.87	0.08	-	1.35
TL6 (cuphea-TE)												
High C22:1 lines	92	-	-	21.1	1.6	17.4	14.7	5.9	1.1	11.6	22.9	-
Low C22:1 lines	68	-	-	29.3	2.1	40.0	16.6	6.7	1.0	1.0	0.0	-
Ratio		-	-	1.39	1.30	2.31	1.13	1.13	0.95	0.09	-	1.52

† The ratio of the sum of the total fatty acids in low C22:1 lines to the sum of fatty acids from C12:0 to C18:3 in high C22:1 lines plus the sum of C20:0 to C22:1 in low C22:1 lines.
‡ Not applicable for the ratio or less than 1% for the fatty acid level.
§ The ratio of the fatty acid level in low C22:1 lines to that in high C22:1 lines.

For example, in the bay-TE transgenic DH lines with low C22:1 levels (less than 1% C22:1), the average C12:0 level was 27.1%, which was 1.45-fold higher than that of the high C22:1 lines (18.7% C12:0, Table 3.9). However, the 1.45 ratio was lower than the expected average ratio 1.51, the ratio by which the other fatty acids in the seed oil would increase as the C22:1 level decreased assuming the regulation of the biosynthesis of the other fatty acids was not changed. The average ratio 1.51 was calculated by dividing the sum (98.4%) of fatty acids from C12:0 to C22:1 in the low C22:1 lines by the sum (63.5%) of the fatty acids from C12:0 to C18:3 plus the background level (1.4%) of very long chain fatty acids (VLCFA), C20:0-C22:1. The total level of the VLCFA in the low C22:1 seeds was considered as the background level. Similarly, the C16:0 level in low C22:1 DH lines expressed the nutmeg- or the cuphea-TE transgenes was increased in a lower proportion than the respective expected average ratios (Table 3.9).

DH Lines from Crosses with Different Non-Transgenic Parents

As noted, the effect of recipient genotype, detected by comparing F_1 seeds from Apollo or AC Excel with F_1 seed from Mercury, could be caused by differences in the C18:3 and C22:1 levels or other genetic differences. Since the C18:3 level was not associated with the variation in the target fatty acid but C22:1 accumulation did have an influence on the target fatty acids as discussed above, the low C22:1 DH lines from Mercury were compared to lines from Apollo and AC Excel to investigate whether there existed other genetic differences between the genotypes, in addition to the C22:1 level, influencing the target fatty acids.

The mean C12:0 level in the low C22:1 lines carrying the bay-TE transgene from Mercury was not significantly different from those in the bay-TE lines from Apollo or AC Excel (Table 3.8). As well, no significant difference were detected in the C16:0 level between the low C22:1 lines from Mercury and the low C22:1 lines from Apollo or AC Excel carrying the nutmeg-TE. The cuphea-TE lines with low C22:1 levels from Mercury produced even higher levels of C16:0 (29.4%, on average) than the lines from Apollo (25.3%) and AC Excel (25.9%). Therefore, it can be concluded that the accumulation of C22:1, rather than other genetic factors, caused the lower levels of the target fatty acids in the high C22:1 DH lines from Mercury.

Transgenic DH lines from crosses with Apollo or AC Excel as the non-transgenic parent were not significantly different in the level of the target fatty acids for the four TE transgenes (Table 3.8). This agrees with the results obtained from F_1 seeds. Therefore, the recipient genotypes of the DH lines originating from the two cultivars as the non-transgenic parent had similar effects on the production of the target fatty acids. Although the two parents differed in the level of C18:3 and likely in other genetic factors, these differences did not influence the target fatty acid level.

3.4.5 Maternal Effect on the Level of the Target Fatty Acids

The effect of maternal plants on the accumulation of the target fatty acids was tested by comparing F_1 seeds from reciprocal crosses (Table 3.10). The reciprocal F_1 seeds were produced by crossing-pollinating the two parents, thus the genotype of the embryo of F_1 seeds developing on the two parental lines was the same, while the maternal sporophyte genotype as well as the cytoplasm of the embryo of the reciprocal F_1 seeds were different.

F_1 seeds from reciprocal crosses between Apollo and each of the four transgenic parental lines did not differ in the target fatty acid level. The C12:0 levels of TL1 x Apollo seeds was similar to that of the reciprocal Apollo x TL1seeds, 37.3% and 36.9%, respectively (Table 3.10). Also, no significant differences were observed for each of the other three pairs of reciprocal crosses, including TL3 x Apollo and Apollo x TL3, TL5 x Apollo and Apollo x TL5, TL6 x Apollo and Apollo x TL6. Therefore, the level of the target fatty acids in the seed oil from these crosses was completely determined by the embryo genotype of the seeds. Apollo or the transgenic lines as the maternal genotype was the same for the accumulation of the target fatty acids in the seed oil.

However, F_1 seeds from the reciprocal crosses with AC Excel as the non-transgenic parent showed maternal effects. AC Excel as the female parent resulted in significantly lower levels of the target fatty acids in the F_1 seeds than the transgenic parental lines as the female in two pairs of reciprocal crosses (Table 3.10). AC Excel x TL1 seeds had a lower level of C12:0 (31.5%) than TL1 x AC Excel seeds (35.5%); AC Excel x TL3 seeds had a lower level of the target fatty acid C16:0 (28.3%) than the reciprocal TL3 x AC Excel seeds (31.2%). For the other two pairs of reciprocal crosses,

Table 3.10 Mean level of the target fatty acids for F_1 seeds from reciprocal crosses of the four transgenic parents with Apollo, AC Excel or Mercury.

Transgenic parent	Cross†	Fatty acid/ parameter‡	Non-transgenic parent		
			Apollo	Excel	Mercury
Bay-TE TL1	TL1 x C	C12:0(%)	37.3	35.5	26.1
	C x TL1	C12:0(%)	36.9	31.5	17.2
		d	0.4	4.0	8.9
		n	3	3	3
		t	0.17	11.71 **	5.75 *
Elm-TE TL3	TL3 x C	C16:0(%)	31.2	31.2	26.6
	C x TL3	C16:0(%)	29.5	28.3	23.8
		d	1.7	2.9	2.8
		n	10	11	10
		t	0.99	2.38 *	1.91
Nutmeg-TE TL5	TL5 x C	C16:0(%)	25.3	24.6	22.8
	C x TL5	C16:0(%)	24.9	26.9	16.0
		d	0.4	-2.3	6.8
		n	6	5	7
		t	0.32	-2.50	5.18 *
Cuphea-TE TL6	TL6 x C	C16:0(%)	29.6	29.9	27.6
	C x TL6	C16:0(%)	27.5	25.9	23.5
		d	2.1	4.0	4.1
		n	10	10	9
		t	1.29	2.02	2.43 *

*, **: Significant at the 0.05 and 0.01 level, respectively, by paired t-test.
† "C" represents the three non-transgenic cultivars, Apollo, AC Excel, or Mercury.
‡ d, the difference between the reciprocal crosses; n, the number of replicates; t, the t-value of paired-t test.

TL5/AC Excel and TL6/AC Excel, no significant difference was detected between the reciprocals, indicating that the cytoplasm and the genotype of TL5 and TL6 were similar to those of AC Excel for the accumulation of the target fatty acid of the nutmeg- and the cuphea-TE transgene.

The reciprocal crosses of Mercury with TL1, TL5 and TL6 also showed a maternal effect (Table

3.10). The F_1 seeds with Mercury as the female parent accumulated lower levels of the target fatty acids than the F_1 seeds with the transgenic lines as the female parent. The C12:0 level of TL1 x Mercury (26.1%), for example, was significantly higher than that of Mercury x TL1 (17.2%). The maternal effect was not related to the C22:1level since the F_1 seeds from the reciprocal crosses between Mercury and the non-transgenic parents did not have significantly different C22:1 levels, e.g. TL1 x Mercury having 19.9% and Mercury x TL1 having 21.4% C22:1, TL6 x Mercury having 18.3% and the reciprocal having 17.3% C22:1.

3.4.6 Cytoplasmic Effect on the Level of the Target Fatty Acids

Transgenic DH lines developed from reciprocal crosses had different cytoplasms, while the other genetic factors, including the transgene, the genotype of the seed embryo and the maternal sporophyte genotype, were the same. Therefore, a significant difference in the mean target fatty acid level between the reciprocal DH lines would be an indication of a cytoplasmic effect.

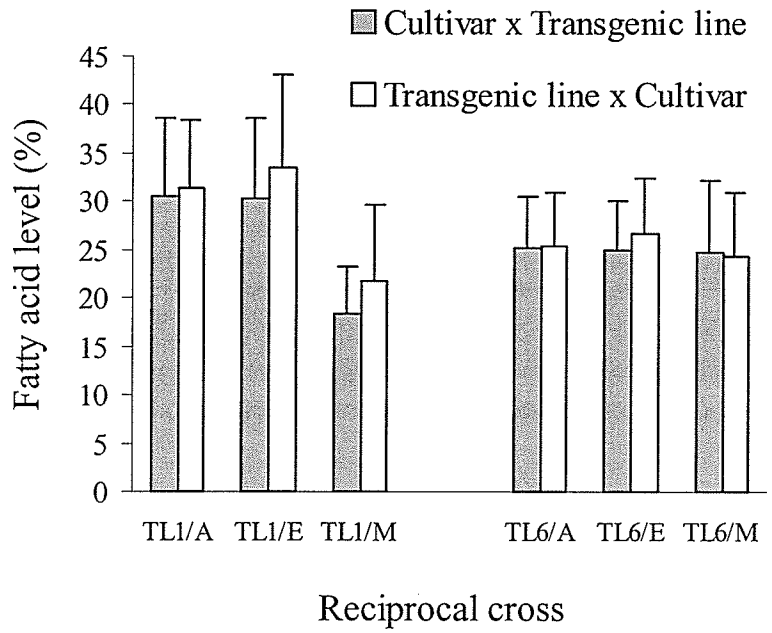


Figure 3.6 Mean levels of the target fatty acids for DH lines from reciprocal crosses of transgenic parents TL1 and TL6 with Apollo (A), AC Excel (E) or Mercury (M). For TL1-crosses, the target fatty acid is C12:0, for TL6-crosses, C16:0. Each of the thick bars represents the mean of at least eight DH lines. Error bars represent the standard deviation.

DH lines developed from the reciprocal crosses TL1/Mercury (incl. TL1 x Mercury and Mercury x TL1, as well as the lines from the reciprocals of TL1/AC Excel, were not significantly different in the C12:0 level (Fig. 3.6). As well, reciprocal DH lines from the three paired reciprocals TL1/Apollo, TL6/Apollo and TL6/AC Excel were also not significantly different in the level of the target fatty acids. These data suggested that the cytoplasm of the three cultivars was not different from the cytoplasm of the transgenic parents for the effect on the accumulation of the target fatty acids.

3.4.7 Interaction of the TE Transgenes

Interaction of the Bay-TE with the Other Three TE Transgenes

Two different TE transgenes was combined into the same seeds by cross-pollinating one of the transgenic parental lines with another. Double-TE seeds that carried the bay-TE transgene and any of the other three C16:0-TE transgenes (the elm-, nutmeg- and cuphea-TE) co-expressed the two different TE, as shown by the accumulation of C12:0 and enhanced levels of C16:0 in the seed oil (Table 3.11).

The level of C12:0, targeted by the bay-TE, was not influenced by the expression of the nutmeg- or cuphea-TE transgenes in the same seeds; e.g. TL1/TL6 seeds, co-expressing the bay-TE and the cuphea-TE showed no significant difference in the C12:0 level (17.6%) from the single-TE seeds (16.2%) with only the bay-TE transgene (i.e. TL1/C1 seeds in Table 3.11). However, co-expression of the bay-TE and the elm-TE in TL1/TL3 seeds resulted in a significantly lower level (11.3%) ofC12:0 in comparison with the bay-TE hemizygous seeds.

On the other hand, the level of C16:0 targeted by the cuphea-TE was not affected by the expression of the bay-TE in the same seeds. The double-TE TL1/TL6 seeds carrying both the bay-TE and the cuphea-TE showed an identical level of C16:0 to the cuphea-TE TL6/C1 seeds (Table 3.11). But the C16:0 levels of TL1/TL3 seeds (16.0%) and TL1/TL5 seeds (8.9%) were significantly lower than the corresponding hemizygous seeds expressing the elm-TE (TL3/C1, 23.3%) and the nutmeg-TE

(TL5/C2, 12.2%), respectively. The results indicated that depending on the nature of a C16:0-TE, the levels of the two target fatty acids in seeds co-expressing the bay-TE and the C16:0-TE may, or may not, be different from those seeds in which the TE being expressed separately.

Interaction between the Elm-TE, Nutmeg-TE and Cuphea-TE Transgenes

Two different C16:0-TE transgenes in the same seeds were also not co-suppressed, as shown by enhanced C16:0 levels in the double-TE seeds compared to the respective hemizygotes of the two TE (Table 3.12). Double-TE TL3/TL5 seeds co-expressing the elm-TE and the nutmeg-TE, for example, produced 20.5% C16:0, which was not significant different from that of the elm-TE hemizygous seeds of TL3/C1 (23/3% C16:0) but significantly higher than that of the nutmeg-TE hemizygous seeds of TL5/C2 (12.2% C16:0).

Different combinations of the TE transgenes resulted in different C16:0 levels in double-TE F_1 seeds. Co-expression of the elm-TE with the cuphea-TE transgene in F_1 seeds of TL3/TL6 led to 26.9% C16:0, which was significantly higher than the C16:0 level in the double-TE seeds of TL3/TL5 co-expressing the elm-TE and the nutmeg-TE (20.5%, Table 3.12). The difference between TL3/TL6 and TL3/TL5 seeds could be accounted for by the difference between TL6 and TL5 since the cuphea-TE homozygous line TL6 produced significantly higher level of C16:0 than the elm-TE homozygous line TL3. Similarly, co-expression of the cuphea-TE with the nutmeg-TE in TL3/TL6 showed a significantly higher level of C16:0 (26.9%) than the C16:0 level in double-TE seeds of TL5/TL6 co-expressing the cuphea-TE and nutmeg-TE (20.6%). The significant difference between TL3/TL6 and TL5/TL6 was related to the difference between TL3 and TL5. The co-expression of the nutmeg-TE with the elm-TE in TL3/TL5 was not significantly different from the co-expression of the nutmeg-TE with the cuphea-TE in TL5/TL6 seeds since the C16:0 levels of the homologous parental lines TL3 and T6 was not significantly different. Thus, there were no indication for differential interaction between these C16:0-TE transgenes observed. In addition, the results showed that

Table 3.11 Mean level (%) of palmitic acid (C16:0) and lauric acid (C12:0) for non-transgenic and transgenic parents, F_1 seeds carrying one of the bay-TE, elm-TE, nutmeg-TE or cuphea-TE transgenes (single-TE F_1 seeds), and F_1 seeds carrying two of the four TE transgenes (double-TE F_1 seeds).

	Parent					Single-TE F1 seeds				Double-TE F1 seeds		
Parameter†	C	TL1	TL3	TL5	TL6	TL1/C1‡	TL3/C1	TL5/C2‡	TL6/C1	TL1/TL3	TL1/TL5	TL1/TL6
C16:0(%)	5.3	4.9	24.7a	14.4bc	25.2a	5.9	23.3a	12.2c	16.5b	16.0b	8.9d	16.5b
C12:0(%)	-§	26.3a	-	-	-	16.2bc	-	-	-	11.3d	12.2cd	17.6b
No. of rep.	10	2	4	4	4	3	4	7	5	4	4	7

† For parents, a single plant was a replicate (rep.); for F1 seeds, a single cross between a male and a female parental plant was a rep. The means followed by the same letters were not significantly different by the Fisher's LSD test at the 0.05 level. The means not followed by any letter was not included in the tests.

‡ "C1", "C2", and "C" represent the non-transformed parents 212/86, QO4 and both, respectively.

§ Less than 1%.

Table 3.12 Mean palmitic acid (C16:0) level (%) of non-transgenic and transgenic parents, F_1 seeds carrying one of the elm-TE, nutmeg-TE or cuphea-TE transgenes (single-TE F_1 seeds), and F_1 seeds carrying two of the three TE transgenes (double-TE F_1 seeds).

	Parent				Single-TE F1 seeds			Double-TE F1 seeds		
Parameter†	C‡	TL3	TL5	TL6	TL3/C1‡	TL5/C2‡	TL6/C1	TL3/TL5	TL3/TL6	TL5/TL6
C16:0(%)	5.3	24.7ab	14.4d	25.2ab	23.3ab	12.2d	16.5cd	20.5bc	26.9a	20.6bc
No. of rep.	10	4	4	4	4	7	5	8	6	8

† For parents, a single plant was a replicate (rep.); for F1 seeds, a single cross between a male and a female parental plant was a rep. The means followed by the same letters were not significantly different by the Fisher's LSD test at the 0.05 level. The means not followed by any letter was not included in the tests.

‡ "C1", "C2", and "C" represent the non-transformed parents 212/86, QO4 and both, respectively.

co-expression of two different C16:0-TE in the same seeds did not increase the C16:0 level compared to the transgenic parent with a higher C16:0 level (Table 3.12). For example, F_1 seeds of TL3/TL6 carrying the elm-TE and the cuphea-TE showed a C16:0 level of 26.9%, not significantly different from the homozygous parental lines TL3 (24.7%) and TL6 (25.2%).

3.5 DISCUSSION AND CONCLUSION

This study focused on influences of other genetic factors, in addition to the TE transgenes themselves, on the level of the fatty acids targeted by the TE in transgenic DH lines. The factors assessed included the (nuclear) recipient genotype, cytoplasm, maternal sporophyte genotype, as well as the presence of an additional different TE transgene.

3.5.1 The Effect of Recipient Genotype on the Substrate Specificity of the TE

There are reports indicating that recipient genotype influences the relative level of the fatty acids targeted by the same TE. *C. hookeriana* accumulates twice as much C8:0 as C10:0 in the seed oil and does not accumulate high levels of fatty acids with a chain length longer than C10:0. However, *B. napus* plants transformed with the TE gene from this species produced more C10:0 than C8:0, and a high level of C16:0 (Jones et al., 1995). Also, no TE gene has been cloned with specificities completely matching the corresponding fatty acid compositions of elm seed oil which predominantly accumulates C14:0; and *B. napus* transformed with the elm-TE accumulated more C16:0 than C14:0. Nutmeg seed oil has high levels of C8:0 and C10:0, while *B. napus* plants transformed with the TE cloned from this species accumulated high levels of C16:0 (Voelker et al., 1997). Such differences in the relative levels of the target fatty acids between the transgenic plants and the original species have also been found by other researchers (Davies, 1993; Dehesh et al., 1996). A possible explanation was that other enzymes, e.g. special KAS, in the recipient genotype influenced the relative levels of the target fatty acids (Voelker et al., 1997).

In the present study the relative levels of the target fatty acids of the same TE were similar when the

TE were expressed in different *B. napus* recipient genotypes that originated from crosses between the transgenic parents and different non-transgenic parents which had different levels of C22:1 and C18:3. Bay-TE transgenic lines always accumulated a much higher level of C12:0 than C14:0, regardless of difference in the non-transgenic parent and in the C22:1 level between the DH lines. Similarly, no influence of recipient genotypes was observed on the specificities in the target fatty acids of the other three TE transgenes. For the cuphea-TE, C16:0 was not changed by the recipient genotype as the major target fatty acid. For the elm- or nutmeg-TE, C16:0 also remained the main target fatty acid in transgenic lines having different recipient genotypes, with much lower levels of other target fatty acids, e.g. C14:0, being detected in all the cases. The relative levels of the target fatty acids of the TE observed in this study were consistent with those as observed in the original transformants of the TE (Jones et al., 1995; Voelker et al., 1996, 1997). These results suggested that, within *B. napus,* the genotypes with distinct fatty acid compositions are similar for some enzymes such as β-ketoacyl-ACP synthase (KAS) which may influence the relative levels of the target fatty acids of TE.

3.5.2 The Effect of Recipient Genotype with Different C22:1 Levels

Expression of the TE in the recipient genotypes with low C22:1 levels (<1%) showed significantly higher levels of the target fatty acids than in the high C22:1 recipient genotypes. The positive effect of low C22:1 recipient genotypes for higher levels of accumulation of the target fatty acids was shown by analyses on F_1 seeds and DH lines with different C22:1 levels that originated from different crosses, as well as on DH lines with contrasting C22:1 levels developed from the same crosses. Comparison of the lines from the same crosses provided more direct evidence for the positive effect of recipient genotypes characterized by a low C22:1 level since the two groups of lines, of high or low C22:1 levels were from the same crosses and assumed to have combinations of the same genes unlinked to the low C22:1 trait.

Higher levels of the target fatty acids in the low C22:1 recipient genotypes than in the high C22:1 genotypes could be accounted for by the reduction in the level of C22:1 in the total fatty acids in the low C22:1 lines. Since the level of a fatty acid was reported as the percentage of the total fatty acids

(Hougen and Bodo, 1973), as the percentage of one fatty acid decreases, the percentages of the other fatty acids would be anticipated to rise if the regulation of the fatty acid synthesis was not changed. Although the low C22:1 DH lines showed higher levels of the target fatty acids than the high C22:1 DH lines, the difference in the target fatty acid level between the low and high C22:1 lines was lower than expected due to the reduced percentage of C22:1 in the total fatty acids in the low C22:1 genotypes. Relative concentrations of the acyl-ACP substrates of the TE have been linked to variation in the target fatty acid levels (Davies, 1993; Voelker et al., 1997). However, it seemed that the reduction in the C22:1 level in the low C22:1 lines did not increase the availability of the acyl-ACP substrates for the target fatty acids in relation to the substrates for the other fatty acids in the seed oil, because the difference in the target fatty acid level between the high C22:1 lines and low C22:1 lines was lower than expected with the reduction of C22:1 in the total fatty acids.

3.5.3 The Effect of Recipient Genotypes with Different C18:3 Levels

No effect of C18:3 accumulation on the target fatty acid levels was detected in this study. F_1 seeds and DH lines that originated from crosses between transgenic lines and the low C18:3 parent Apollo or the high C18:3 parent AC Excel showed similar levels of the target fatty acids. Especially, the low and high C18:3 DH lines developed from the same crosses were not significantly different from each other in the target fatty acid level.

3.5.4 Maternal and Cytoplasmic Effects

The levels of the target fatty acids were determined by the genotype of the embryo in the seeds because the F_1 seeds (developed from cross-pollination with transgenic parents) on non-transgenic parents produced significant levels of the target fatty acids. In this regard, the genetic control of the fatty acids targeted by the TE transgenes was not different from that of the fatty acid compositions of non-transgenic oilseed plants shown in some genetic studies. Such examples include the C22:1, C18:1 and C18:2 levels in *B. napus* (Downey and Harvey, 1963; Thomas and Kondra, 1973) and the C16:0 and C18:0 levels in soybean (*Glycine max*) (Kinoshita et al., 1998; Narvel et al., 2000; Rahman et al., 1997).

However, similar to that observed in some studies with the C18:1, C18:2 and C8:3 levels of non-transgenic *B. napus* plants (Pleines and Friedt, 1989, Rakow, 1973; Thomas and Kondra, 1973) and the C18:0 level of non-transgenic sunflower plants (Pérez-Vich et al., 1999), the levels of the target fatty acids were not completely under embryo genetic control. Some reciprocal crosses between the transgenic parental lines and AC Excel or Mercury showed significant differences although the embryo nuclear genotypes in reciprocal F_1 seeds were the same. The difference between reciprocal F_1 could be caused by different sporophyte genotypes of the two parents (maternal effect) and /or different cytoplasm (cytoplasmic effect) (Thomas and Kondra, 1973). Since the DH lines developed from reciprocal crosses were not significantly different, cytoplasmic effect on the target fatty acid levels was excluded. Some agronomic or physiological characters have been associated with variation in the fatty acid composition of non-transgenic rapeseed oils (Pleines and Friedt, 1989; Thomas and Kondra, 1973; Wilmer et al., 1996). For instance, phenotypic differences in the time of flowering would lead to seed development under different environmental conditions. Therefore, the maternal effect observed with F_1 seeds might represent the difference in such traits between the transgenic parental lines and the two cultivars, AC Excel and Mercury.

3.5.5 Interaction of TE Transgenes

Homology-dependent gene silencing (HDGS) phenomenon, which is based on recognition of nucleic acid sequence homology between the interacting genes (Matzke et al., 1999; Meyer and Saedler, 1996), has been described in diverse organisms (Matzke et al., 1999). HDGS can occur due to interaction between repeats located at the same site or different sites (Matzke et al., 1994). The promoter and terminator of the four TE transgenes were the same (Jones et al., 1995; Voelker et al., 1996, 1997), which are 1.7 and 1.2 kb long, respectively (Kridl et al., 1991). Moreover, these TE genes share high homology in the DNA sequences (Jones et al., 1995), e.g. the bay- and cuphea-TE sequences average 61% identity in part (ca. 1 kb) of the coding regions (Jones et al., 1995). However, transgenic seeds containing the bay-TE and any of the other three TE transgenes co-expressed the two TE. Thus, introduction of an additional TE with homology to the previous ones does not necessarily lead to co-suppression. High levels of expression in transgenic plants carrying various transgenes with multiple copies of the same transgene have been widely reported (Gendloff

et al., 1990; Hobbs et al., 1993; McCabe et al., 1999; van der Hoeven et al., 1994), including *B. napus* plants transformed with a construct containing two copies of the same TE gene (Voleker et al., 1996).

Co-expression of two TE could result in accumulation of the target fatty acids of the two TE at the same level as found in seeds which expressed the TE individually. In transgenic seeds carrying both the bay-TE and the cuphea-TE transgenes, for example, the levels of the target fatty acids C12:0 and C16:0 of the two TE were not significantly different from the C12:0 level of the bay-TE hemizygous seeds and the C16:0 level of the cuphea-TE hemizygous seeds, respectively. Therefore, co-expression of two different TE transgenes can be used as an additional approach to develop *B.napus* cultivars with a modified fatty acid composition of the seed oil, e.g. oils with high levels of both C12:0 and C16:0.

CHAPTER 4

EFFECTS OF GENOMIC POSITION AND COPY NUMBER OF ACYL-ACP THIOESTERASE TRANSGENES ON THE LEVEL OF THE TARGET FATTY ACIDS IN *BRASSICA NAPUS* L.

4.1 ABSTRACT

The effects of genomic position and copy number of acyl-acyl carrier protein (ACP) thioesterase (TE) transgenes on the levels of the major target fatty acids, lauric acid (C12:0) or palmitic acid (C16:0) depending on the TE, were investigated in this study. The TE transgenes encode the bay-TE (Uc FatB1), elm-TE (Ua FatB1), nutmeg-TE (Mf FatB1) and cuphea-TE (Ch FatB1), respectively. F_1's of four crosses were produced by crossing four TE transgenic parents, transformed individually with the TE genes, with non-transgenic parental lines '212/86' or 'QO4', the recipient genotypes used for the original transformation of the transgenic parental lines. The F_1 seeds had half the number of the transgene copies compared to self- pollinated (SP) seeds of the respective transgenic parental lines. F_1 seeds carrying the bay-TE or the cuphea-TE transgenes showed significantly lower levels of the target fatty acids of the TE than the SP seeds of the corresponding transgenic parents. Doubled haploid (DH) lines were developed through microspore culture from the hybrids between the elm-TE or cuphea-TE transgenic parental lines and non-transgenic plants. DH lines carrying one to five copies of the cuphea-TE transgene displayed a positive linear correlation between the copy number and the level of the target fatty acid C16:0 level ($r = 0.77^{**}$). DH lines with five copies produced 26.1% C16:0 on average, compared to the lines with only one copy (15.7%). DH lines with the cuphea-TE transgene at two different loci were not significantly different in the C16:0 level. However, DH lines with the elm-TE transgene at four different loci showed significantly different C16:0 levels, with one of the loci (Locus E-II) leading to a significantly higher level of C16:0. DH lines with more copies than the DH lines with Locus E-II did not show an enhanced C16:0 level. These results indicate that higher levels of the target fatty acid of a TE transgene could be achieved

by increasing the copy number and/or selecting plants with the TE transgene at different genomic positions.

Abbreviations: ACP, acyl carrier protein; C12:0, lauric acid;C16:0, palmitic acid; DH, doubled haploid; DIG, digoxygenin; SP, self-pollinated; TE, acyl-acyl carrier protein (ACP) thioesterase.

4.2 INTRODUCTION

The seed oil of conventional canola cultivars (*B. napus* L.) has a fatty acid composition with traces of lauric acid (C12:0), ca. 4% palmitic acid (C16:0), and more than 90% C18 fatty acids which include stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Eskin et al., 1996; McVetty and Scarth, 2002; Voelker et al., 1996). Transformation of canola plants with special TE genes cloned from several non-crop plant species has resulted in the development of transgenic lines accumulating C12:0 or enhanced level of C16:0 in the seed oil (Jones et al., 1995; Voleker et al., 1996, 1997). The TE can hydrolyze the thioester bond between the elongating fatty acyl group and the co-factor acyl carrier protein (ACP), thus leading to premature termination of the fatty acid biosynthetic process and accumulation of fatty acids with a shorter chain length in the seed oil.

Wide variation in the levels of the fatty acids targeted by TE was frequently observed among transgenic plants from independent transformation events in the same transformation experiments. Among transformants with the bay-TE transgene (*Uc FatB1*), for example, the level of the target fatty acid, lauric acid (C12:0), varied from the background level (near zero) to up to 40% (Voelker et al., 1996). The C18:0 level of independent transformants carrying a FatA TE (*Garm FatA1*), cloned from a mangosteen seed cDNA library, varied from the background level (ca. 2%) to 22%. (Hawkins and Kridl, 1998). Wide variation was also observed among plants transformed with other genes involved in the plant fatty acid biosynthetic pathway, e.g. genes coding for desaturases (Hitz et al., 1995; Knutzon et al., 1992).

The effects of genomic position and copy number of transgenes on the expression level have been reported in plants (Allen et al., 2000; De Neve, 1999; Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999). Since foreign DNA integrates almost exclusively at random, non-homologous sites during transformation, some integrations may occur in transcriptionally active chromatin environments, others in condensed, transcriptionally inert chromatin regions (Mengiste and Paszkowski, 1999). It is believed that transgenes in heterochromatic areas such as those surrounding centromeres are prone to silencing and give rise to reduced and/or variable expression (Allen et al., 2000; Maqbool and Christou, 1999; Mengiste and Paszkowski, 1999; Weiler and Wakimoto, 1995). Literature about the relationship between copy number and expression level of transgenes is conflicting (Hobbs et al., 1993; McCabe et al., 1999). The two variables could be negatively correlated (Cervera et al., 2000; Hobbs et al., 1993; Mannerlöf et al., 1997), not correlated (Bauer et al., 1998; Hobbs et al., 1993; McCabe et al., 1999), or positively correlated (Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999; van der Hoeven et al., 1994). In many reports, the results were mainly based on analyses of transgenic plants from independent transformation events (Cervera et al., 2000; Gendoloff et al., 1990; Hobbs et al., 1990; Mannerlöf et al., 1997).

In the present study, the effects of the genomic position and copy number of TE transgenes on the levels of the fatty acids targeted by the TE were studied by comparing self-pollinated (SP) seeds and F_1 seeds carrying the same TE for each of four TE transgenes, as well as by analysis of DH lines with different transgene loci and copy numbers for each of two TE transgenes.

4.3 MATERIALS AND METHODS

4.3.1 Parental Genotype and Production of F_1 and SP Seeds

The original seeds of four transgenic parental lines TL1, TL3, TL5 and TL6, as well as two non-transgenic *B. napus* breeding lines 212/86 and QO4, were kindly provided by Calgene Inc. (USA). TL1 and TL5 were homozygous for one transgene locus; TL3 and TL6 had three or more transgene loci (M. Sovero, personnal comm.). TL1, developed by *Agrobacterium*-mediated transformation

with the bay-TE transgene *Ua FatB1* (Voelker et al., 1992, 1996), showed ca. 40% C12:0 in the original seeds. TL3, TL5 and TL6, transformed with the elm-TE *Uc FatB1*, nutmeg-TE *Mf FatB1* (Voelker et al., 1997), and cuphea-TE *Ch FatB1* (Jones et al., 1995), respectively, showed ca. 30-35% C16:0 in the original seeds. The breeding line 212/86 was the genotype used in the transformation for the development of TL1, TL3 and TL6, and QO4 was the genotype for TL5. Like 212/86 and QO4, the three non-transgenic *B. napus* cultivars, Apollo (Scarth et al., 1995a), AC Excel (Rakow, 1993) and Mercury (Scarth et al., 1995b) all have only ca. 4% C16:0 and no accumulation of C12:0 in the seed oil.

The four transgenic parental lines together with 212/86 and QO4 were grown in a growth room with day/light temperatures of 25/20 °C and a 16-h photoperiod and 580 μE m^{-2} s^{-1} light intensity. F_1 seeds of four crosses were produced by crossing the four transgenic parents as the female with the male being the non-transgenic genotype used in the original transformation of the transgenic lines, 212/86 or QO4. F_1 seeds and SP seeds were harvested from the individual parental plants at maturity and the fatty acid composition was determined as described below.

4.3.2 DH Line Development and Planting

The DH lines were developed by microspore culture followed by chromosome doubling based on the procedure described by Ferrie and Keller (1995). Microspores were isolated from unopened flower buds of transgenic hybrid plants that originated from crosses of the elm-TE or cuphea-TE parental lines as the female parents and Apollo, AC Excel or Mercury as the male parents as described above. Embryogenesis was induced by culturing the microspores in induction media at 32.5 °C for 3d. DH_1 plants, transgenic or non-transgenic, were self-pollinated to produce DH_2 seeds. The DH_2 seeds were tested for the fatty acid composition. Transgenic lines carrying the TE transgenes, as characterized by enhanced levels of C16:0, were grown in a greenhouse and self-pollinated to obtain DH_3 seeds.

To study the effects of the genomic position and copy number, DH_3 seeds of 128 transgenic DH lines, including 101 cuphea-TE transgenic DH lines and 27 elm-TE transgenic DH lines and eight

non-transgenic DH lines (included as the control) were grown in 15-cm pots, with one plant each line, in a growth room. The growth room had day/night (16h/8h) temperatures of 20/15 °C and light intensity as described above. SP DH_4 seeds were harvested from each of the DH_3 plants and tested for the fatty acid composition, locus and copy number, as described below.

4.3.3 Preparation of DNA Probes

The probes were prepared by a digoxygenin (DIG)-labeling procedure with a PCR DIG probe synthesis kit (Roche, Germany). To prepare the cuphea-TE probe, the TE transgene was amplified from genomic DNA of the transgenic parental line with the left primer PT1, 5'-ATTAGAGCCTCGGCTTCACTC-3' and the right primer PT2, 5'-GGATCCCATTGGATGATCTTT-3' by PCR. The amplified DNA fragment was cloned with the pGEM®-T Easy Vector and JM109 Competent Cells (Promega, Madison, USA). A positive white colony was grown overnight in LB Broth medium and the plasmid DNA carrying the cuphea-TE gene was extracted (Ausubel et al., 1995). With the plasmid DNA having the cuphea TE gene as the template, an 1.1-kb internal fragment of the cuphea-TE transgene was produced and DIG-labeled by PCR under the presence of DIG-dUTP. With the same procedure, a 0.9-kb probe for the elm-TE transgene was prepared with the primer E3, 5'-TCCACAACAGCACCATCATT-3' and primer E2, 5'-CTTGCTGCAATCACGACTGT-3', for the PCR DIG labelling reaction.

4.3.4 Plant Genomic DNA Isolation and Southern Blotting

Plant genomic DNA was extracted from ca. 3g of cotyledon and young leaves using a CTAB procedure (Kidwell and Osborn, 1992). Extracted DNA was purified with 25:24:1 of chloroform, phenol and isoamyl alcohol (Ausubel et al., 1995). Genomic DNA (ca. 5ug) was digested with *Nsi*I, separated by electrophoresis on 0.8% agarose gel, and blotted onto a nylon hybridization transfer membrane (Hybond-N+, Amersham). *Nsi*I has an unique site located in the napin promoter in the T-DNA region. The T-DNA engineered with the elm- or the cuphea-TE genes was ca. 7.6 and 7.8 kb long, respectively (Jones et al., 1995; Voelker et al., 1997). The T-DNA region from the *Nsi*I site to the left border, which contained the cuphea- or the elm-TE transgenes, was 6.0 and 5.9 kb, respectively. Hybridization and detection was conducted with a DIG non-radioactive hybridization and chemiluminescent detection system by following the product instruction (Roche, Germany).

Hybridization took place overnight in a hybridization buffer, DIG Easy Hyb solution, at 50 °C under the presence of the TE probe, with stringency washes at 68 °C in 2 x Wash solution (2 x SSC, containing 0.1 SDS) for 2 x 30 min. After washes and blocking, the membrane was incubated with a dilution of Anti-Digoxigein-Fab fragment conjugated to alkaline phosphatase (AP). Followed by treatment with an 1:100 dilution of the AP substrate CDP-Star, the luminescent signal was recorded with X-ray films. Since the restriction enzyme had only one site at one end of the TE transgene in the T-DNA region, a plant genomic restriction site was necessary to enable the production of a *Nsi*I fragment carrying the transgene copy. Thus the number of bands would indicate the copy number (Voelker et al., 1996). The sizes of the bands were estimated based on the 1 KB PLUS DNA Ladder (GibcoBRL, Canada) running alongside the digested DNA samples on the agarose gel.

4.3.5 Determination of the Fatty Acid Composition

The fatty acid composition of seed oils was determined by gas chromatography of the methyl ester derivatives of the fatty acids (Hougen and Bodo, 1973). A sample of 10 seed was picked randomly from each of the plants to be tested, and the oil of the 10 seeds was extracted overnight with 1 ml heptane. Then, 300 ul of 0.5M sodium methoxide was added for methyl ester derivitization. The oven temperature was programmed to increase from 190 to 230 °C. The level of a fatty acid was reported as the percentage of the total fatty acids in the seed oil (Chen and Beversdorf, 1990; Knutzon et al., 1992; Pleines and Friedt, 1989; Sommerville, 1993).

4.3.6 Statistical Analysis

Correlation analyses between copy number and expression level and multiple comparisons between means were performed as described by Ott (1993).

4.4 RESULTS

4.4.1 Effect of Copy Number on the Level of the Target Fatty Acids in SP and F_1 Seeds

The mean level of the target fatty acids, C12:0 for the bay-TE and C16:0 for the other three TE transgenes was determined for SP seeds of the four transgenic parental lines and for F_1 seeds of the

four crosses between the four transgenic parental lines and the non-transgenic breeding lines (Table 4.1). The number of transgene copies in F_1 seeds was half that in the SP seeds because the F_1 seeds was produced by cross-pollinating the transgenic lines with non-transgenic plants. Therefore, a comparative analysis of the SP and F_1 seeds provides evidence for the effect of the copy number on the level of the target fatty acids of the TE.

SP seeds carrying the bay-TE or the cuphea-TE showed a significantly higher level of the target fatty acid, C12:0 or C16:0, respectively, than the corresponding F_1 seeds (Table 4.1). Since the F_1 and SP seeds of the same TE are expected to have the same recipient genotype, the difference in the level of the target fatty acids between the SP and the F_1 seeds should be caused by the different numbers of the copies rather by different recipient genotypes. For the elm-TE and the nutmeg-TE transgenes, no significant differences were detected between the SP seeds and the F_1 seeds, indicating that extra copies in the SP seeds did not negatively affect the production of the target fatty acid C16:0.

Table 4.1 Mean levels (%) of the target fatty acids in F_1 seeds from crosses of transgenic parental lines TL1, TL3, TL5 and TL6 with non-transgenic *B.napus* genotypes 212/86 or QO4 and in self-pollinated (SP) seeds of the transgenic parents.

Cross	Parameter†	SP seeds	F_1 seeds	Transgene
TL1 x 212/86	C12:0(%)	26.3 a‡	16.2 b	Bay-TE
	rep.	2	3	
TL3 x 212/86	C16:0(%)	24.7 a	23.3 a	Elm-TE
	rep.	4	4	
TL5 x QO4	C16:0(%)	14.4 a	12.2 a	Nutmeg-TE
	rep.	4	7	
TL6 x 212/86	C16:0(%)	25.2 a	16.5 b	Cuphea-TE
	rep.	4	5	

† For SP seeds, a parental plant was a replicate (rep.); for F_1 seeds, a cross between a male and a female parental plant in pair was a rep.

‡ The means followed by the same letter within the same row are not significantly different by Fisher's LSD test at the 0.05 level.

4.4.2 Segregation of Locus and Copy Number in Cuphea-TE DH Lines

Southern blotting analyses were conducted for 109 DH lines developed from crosses between the cuphea-TE parental line TL6 and the non-transgenic cultivars, which included 101 lines accumulating enhanced levels of palmitic acid (C16:0) targeted by the cuphea-TE transgene. The C16:0 levels ranged from 9.4% to 34.5%. Since non-transgenic canola plants have only ca. 4% C16:0, these lines were putatively transgenic. As well, eight lines with 3.3-5.1% C16:0 were tested as negative control for the transgene.

As expected, all the 101 lines with enhanced levels of C16:0 showed at least one band with the cuphea-TE gene as the probe, and all the control lines showed no band, e.g. plants No. 1485 and 1156 (Fig. 4.1). Five bands of different lengths were detected with the transgenic lines, which were approximately 11.5, 9.0, 8.5, 8.1, 4.0 kb long, and were referred to as Copy 1, 2, 3, 4 and 5, respectively. Copy 1, 3 and 4, co-segregated; e.g., DH lines 697, 742, 1277 all displayed the three

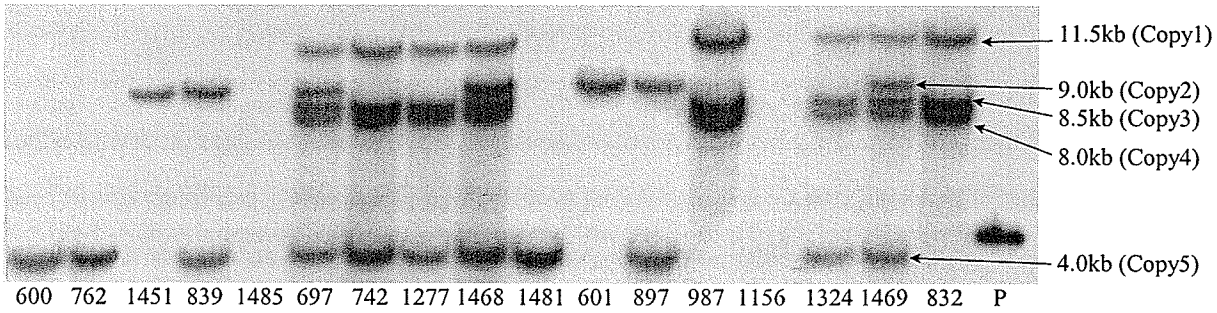


Figure 4.1 Southern blotting analysis of DH lines developed from crosses between the cuphea-TE transgenic parental line TL6 and non-transgenic cultivars Apollo, AC Excel or Mercury. The identification number of the DH lines is presented below the lanes. P represents a digested plasmid carrying the cuphea-TE transgene. Estimated sizes of the bands are shown in kilobase (kb).

copies. None of the 101 transgenic DH lines showed only one or two of these three copies. Therefore it can be concluded that these three copies were closely linked or tandem repeats at a genomic site. The putative site with the three TE transgene copies was designated Locus C-I. Copy 2 and 5 segregated from each other as well as from the other three copies as shown by the DH lines

with only Copy 2 (e.g. DH line No. 1451 and 601) or only Copy 5 (No. 600 and 762), were found. This indicates that Copy 2 and 5 were located at two additional genomic sites, designated Locus C-II and C-III, respectively.

Segregation analysis was conducted to determine whether the three transgene loci segregated independently or were linked. Assuming Locus C-I, C-II and C-III were completely independent, the population of DH plants developed from hybrid plants hemizygous for the three loci should be made up of eight genotypic classes of plants, with seven transgenic genotypic classes and one non-transgenic genotypic class. In addition, DH plants of each of the transgenic genotypes should occur in an equal frequency in the population. The observed frequencies of the seven transgenic genotypes among 75 DH lines, originated from a single transgenic parental plant (TL6.12), were 10, 11, 5, 7, 17, 13, 12, respectively (Table 4.2). These frequencies were not significantly different from the

Table 4.2 Segregation of the cuphea-TE transgene in DH lines developed from crosses of the cuphea-TE transgenic parental line TL6 and non-transgenic cultivars Apollo, AC Excel or Mercury.

	Genotype†								
	Loci:	C-I			C-II	C-III			
Genotypic class	Copy: 1	3	4		2	5	No. of copies	Expected ratio	Observed frequency	X^2
1	1‡	1	1		-‡	-	3	1/7	10	0.04
2	-	-	-		1	-	1	1/7	11	0.04
3	-	-	-		-	1	1	1/7	5	1.18
4	1	1	1		1	-	4	1/7	7	1.90
5	1	1	1		-	1	4	1/7	17	5.86
6	-	-	-		1	1	2	1/7	13	0.14
7	1	1	1		1	1	5	1/7	12	0.01
8§	-	-	-		-	-	0	Not planted		
Total								1	75	8.72¶

† The three distinct loci, C-I, C-II and C-III, were defined based on Southern blotting analyses. Locus C-1 had three copies, Copy 1, 3 and 4; Locus C-II, Copy 2; Locus C-III, Copy 5.

‡ "1", the copy present; "-", the copy absent.

§ DH lines which did not show an enhanced level of C16:0 were not tested except for a few being tested as the control.

¶ The X^2 value 8.72 is not significant. X^2 (0.05,6) = 12.592.

expected equal frequency with three independently segregating loci by X^2-test ($p = 0.19$), supporting the conclusion that the three TE transgene loci were segregating independently.

Based on the Southern blotting results and segregation analysis, it can be concluded that the five cuphea-TE transgene copies, detected in the population of the cuphea-TE DH lines, were present at three independently segregating loci, designated as Locus C-I, C-II and C-III, with Locus C-II and C-III each having only one copy of the cuphea-TE transgene. Locus C-I had three closely linked or tandem repeated transgene copies, which behaved as a single locus.

4.4.3 Effect of Genomic Position in Cuphea-TE DH lines

A total of 83 DH lines, developed from crosses with Apollo or AC Excel as the non-transgenic parent, were divided into seven transgenic genotypic classes based on the Southern blotting results, and the mean C16:0 level of each class was determined (Table 4.3). Of the 83 DH lines, the mean C16:0 level of the 56 DH lines from Apollo was 20.2%, not significantly different from that of the 27 DH lines from AC Excel (19.9%, indicating that Apollo and AC Excel as the non-transgenic parents were not significantly different.

Expression of the cuphea-TE transgene at Locus C-II was not significantly different from the expression at Locus C-III based on the C16:0 level of the DH lines (Table 4.3). The mean C16:0 level of the 15 transgenic DH lines with one copy at Locus C-II was the same statistically as the mean C16:0 level of the 7 DH lines with one copy at Locus C-III (15.5% and 16.1%, respectively). DH lines in Class 4 and 5 had four transgene copies but with different distribution in the genome. Lines in Class 4 each had a copy at Locus C-II and lines in Class 5 each had a copy at Locus C-III, with the lines in the both classes having the three copies at Locus C-1. These two classes had almost the same mean level of C16:0.

The DH lines with only Locus C-I showed a higher mean level of C16:0 than the DH lines with only Locus C-II (15.5%) and the lines with only Locus C-III (Table 4.3). However, it could not be determined, based on the comparison here, whether the distinct genomic position of Locus C-I was

Table 4.3 Mean palmitic acid (C16:0) level (%) of transgenic DH lines with different loci and copy numbers of the cuphea-TE transgene developed from crosses of the culpea-TE transgenic parent TL6 with non-transgenic cultivars Apollo or AC Excel.

Class No.	1	2	3	4	5	6	7	8
Locus†	C-I	C-II	C-III	C-I+C-II	C-I+C-III	C-II+C-III	C-I+C-II+C-III	Control
No. of copies	3	1	1	4	4	2	5	0
No. of DH lines	11	15	7	6	16	18	10	8
C16:0(%)‡	19.4cd	15.5e	16.1de	21.3bc	22.8b	19.8c	26.1a	4.1

† The three distinct loci, C-I, C-II and C-III, were defined based on Southern blotting analyses. Locus C-1 had three copies, Copy 1, 3 and 4; Locus C-II had Copy 2; Locus C-III had Copy 5.

‡ The means followed by the same letters were not significantly different by Fisher's LSD test at the 0.05 level, with non-transgenic control plants not being included in the statistical test.

the reason for the higher level of C16:0 or whether the higher level was the result of the two extra copies at this locus.

4.4.4 Effect of Copy Number in Cuphea-TE DH Lines

Individual DH lines with the same copy numbers of the cuphea-TE transgene showed wide variation in the C16:0 level as shown by the scatter plot of the DH lines (Fig. 4.2). The C16:0 levels of the 22 DH single-copy DH lines, i.e DH lines in Class 2 and 3 (Table 4.3), ranged from 10.6% to 21.2%, a 2-fold difference. DH lines with 2 to 5 copies showed a ca. 1.5-fold difference among lines with the same copy number (Fig. 4.2).

Although there was a wide variations among the DH lines within each class of copy number, the mean C16:0 level of the DH lines increased as the copy number increased (Fig. 4.2). The DH lines each with one copy showed a mean C16:0 level of 15.7%, the weighted average of DH lines in Class 2 and 3 (Table 4.3). The mean level of C16:0 was increased to 19.8%, 22.4%, and 26.1%, respectively, with two, four and five copies. Correlation analysis between copy number and C16:0 level of the 83 DH lines showed that the two variables were significantly positively correlated (r =

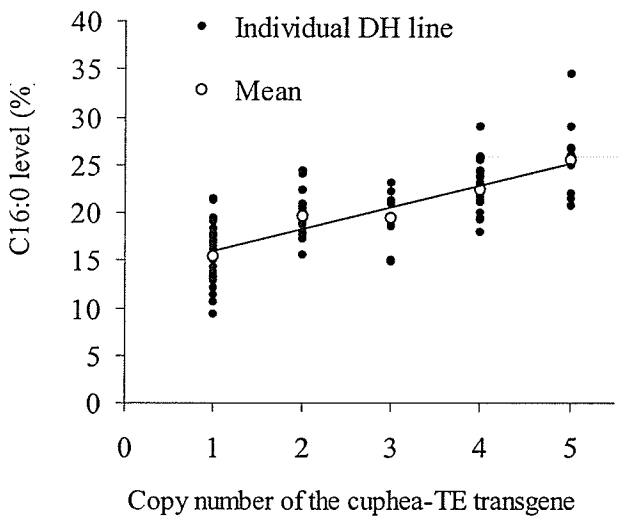


Figure 4.2 Palmitic acid (C16:0) level (%) of DH lines with different copy numbers of the cuphea-TE transgene developed from crosses of the cuphea-TE transgenic parent TL6 with non-transgenic cultivars Apollo or AC Excel.

0.77**).

In addition, the maximum C16:0 level increased with increased copy number in the DH lines (Fig, 4.2). The highest C16:0 level found among the 22 DH lines with one copy was 21.2%. The highest C16:0 level increased to 24.5, 25.8 and 34.5%, respectively, in DH lines with two, four and five copies.

4.4.5 Segregation of Locus and Copy Number in Elm-TE DH Lines

A total of 27 DH lines with the elm-TE transgene were tested for the locus and copy number by Southern blotting analysis. Seven distinct bands, 15.0, 13.0, 11.5, 11.0, 9.0, 8.5 and 8.0 kb in size, were detected, and were referred to as bands 1-7, respectively (Fig. 4.3). The non-transgenic genotype 212/86 did not show any of these bands. Based on the banding patterns, the 27 DH lines were divided into eight groups of distinct transgene genotypes; e.g., the three DH lines in Group 1 showed only band 1(15.0 kb) and the lines in Group 2 showed only bands 2 and 3 (Table 4.4).

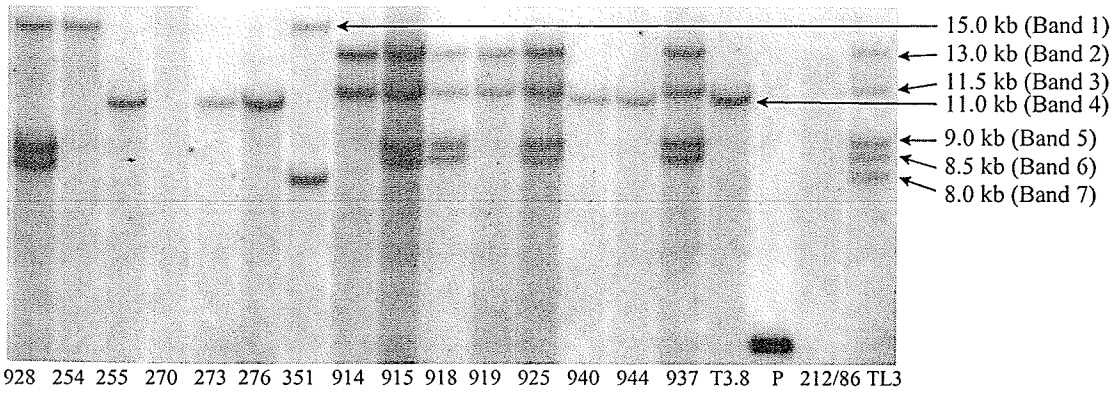


Figure 4.3 Southern blotting analysis of DH lines developed from crosses of the elm-TE transgenic parental line TL3 with non-transgenic cultivars Apollo, AC Excel or Mercury. Identification numbers of the DH lines are presented under the lanes. P is a digested plasmid cloned with the elm-TE transgene; 212/86, a non-transgenic breeding line. Estimated sizes of the bands are shown in kilobase (kb).

Table 4.4 Southern blotting analyses of transgenic DH lines developed from crosses of the elm-TE transgenic parental line TL3 and non-transgenic cultivars Apollo, AC Excel or Mercury.

		Banding pattern and loci†						
	No. of	E-I	E-II		E-III	E-IV		E-V
Group	DH lines	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7
1	3	1‡	-‡	-	-	-	-	-
2	3	-	1	1	-	-	-	-
3	4	-	-	-	-	1	1	-
4	1	1	-	-	-	1	1	-
5	6	-	1	1	-	1	1	-
6	8	-	-	-	1	-	-	-
7	1	-	-	-	-	-	-	1
8	1	1	-	-	-	-	-	1

† The five distinct loci, E-I to E-V, were defined based on the Southern blotting analyses, with E-I, E-III and E-V had one copy of the elm-TE transgene, E-II and E-IV had two copies. The estimated sizes of the bands on Southern blots representing these copies, from Band 1 to Band 7, were ca. 15.0, 13.0, 11.5, 11.0, 9.0, 8.5 and 8 kb, respectively.

‡ "1", the band present; "-", the band absent.

The seven bands represented five elm-TE transgene loci. Band 1 segregated from the other bands, with 3 lines displaying only this band (Table 4.4), e.g. plant No. 254 (Fig. 4.3), indicating that the

elm-TE transgene copy represented by this band was at a genomic site different from the others, and was designated Locus E-I. Bands 2 and 3 were closely linked, showing no segregation. Nine lines showed these two bands (Groups 2 and 5, Table 4.4) and the others did not show either band. This suggested that the transgene copies represented by these two bands were located at a distinct genomic site, designated Locus E-II. Based on similar analyses, the copy represented by band 4 was also concluded to be at a distinct genomic site, designated Locus E-III; bands 5 and 6 represented linked copies at another genomic site designated Locus E-IV. Only two DH lines showed band 7 (Classes 7 and 8), with one line (Class 7) displaying only this band, indicating the transgene copy represented by this band was at a different site, designated Locus E-V.

4.4.6 Effect of Genomic Position in the Elm-TE DH Lines

The mean C16:0 levels of five groups of elm-TE DH lines are presented in Table 4.5. All the lines in groups 1, 2, 3, 5 originated from the cross between the elm-TE transgenic parental line TL3 and the non-transgenic parent AC Excel. Of the six lines in Group 6, four lines originated from Apollo as the non-transgenic parent and two lines from AC Excel as the non-transgenic parent. The mean C16:0 level of the four lines from Apollo was not significantly different from that of the two lines from AC Excel (25.8% and 23.7%, respectively).

All of the DH lines with only one of the four loci, Locus E-I, E-II, E-III or E-IV, showed higher C16:0 level than the non-transgenic control (ca.4%), e.g., the three DH lines with Locus E-I had 10.8% C16:0 on average, indicating that the elm-TE transgene was expressed at any of all the four loci.

The influence of genomic position on the target fatty acid was observed in the elm-TE DH lines. DH lines with the TE transgene at different genomic positions showed significantly different levels of C16:0 (Table 4.5). The mean C16:0 level of the DH lines with only Locus E-I (10.8%) was significantly lower than the mean C16:0 level of the DH lines with only Locus E-III (25.2% on average) although each of the two loci had one copy of the elm-TE transgene. In addition, the mean C16:0 level of the DH lines with only Locus E-II (Group 2) was significantly different from that of

the DH lines with only Locus E-IV (Group 3) although the two loci had the same copy number.

Table 4.5 Mean palmitic acid (C16:0) level for five groups of transgenic DH lines with different loci and copy numbers of the elm-TE transgene.

Group†	Locus‡	Copy Number	C16:0(%)§	No. of DH lines
1	E-I	1	10.8 c	3
2	E-II	2	36.7 a	3
3	E-IV	2	28.7 b	4
5	E-II + E-IV	4	35.2 a	6
6	E-III	1	25.2 b	6

† The identification No. of the groups, 1 to 6, were consistent with Table 4.4.
‡ The four distinct loci, E-I to E-IV, were defined based on the Southern blotting analyses, with E-I and E-III had one copy of the elm-TE transgene, E-II and E-IV had two copies.
§ The means followed by the same letter were not significantly different by Fisher's LSD.

4.4.7 Effect of Copy Number in Elm-TE DH Lines

As the copy number of the elm-TE transgene increased from one to two, the mean C16:0 level of the transgenic DH lines was significantly increased from 10.8% with one copy at Locus E-I to 28.7% with two copies at Locus E-IV or 36.7% with two copies at Locus at E-II (Table 4.5). The mean C16:0 level of the two-copy DH lines with Locus E-II was also significantly higher than the C16:0 level of the single-copy DH lines with Locus E-IV. These results indicated that the extra copies at Locus E-II and E-IV did not negatively affect the accumulation of the target fatty acid C16:0. However, it was not clear whether the higher levels of C16:0 in the DH lines with two copies at Locus E-II or E-IV, compared to single copy lines, were caused by the extra transgene copies or the different genomic positions.

When the copy number of the elm-TE transgene increased from two to four, the C16:0 level did not necessarily increase significantly, depending on the loci involved (Table 4.5). The mean C16:0 level of the DH lines with four copies at Locus E-II and E-VI (35.2% on average) was significantly higher than the mean C16:0 level of the lines with only two copies at Locus E-VI (28.7%), but was not

significantly different from that of the DH lines with two copies at Locus E-II (36.7%). This indicated that when a locus leading to a higher C16:0 level (e.g. Locus E-II) was present, addition of extra copies of the elm-TE transgene had no significant effect on the production of the target fatty acid. When a locus with a lower expression level (e.g. Locus E-III) was present, additional elm-TE transgene copies could significantly increase the C16:0 level.

4.5 DISCUSSION

4.5.1 Effect of Genomic Position

Three independently segregating cuphea-TE transgene loci were identified in the cuphea-TE transgenic DH lines. Two of the loci (Locus C-II and C-III), each having a single copy of the cuphea-TE transgene, were not significantly different based on comparative analysis of the level of the target fatty acid in the transgenic DH lines carrying Locus C-II or C-III. However, the DH lines with the elm-TE transgene at different genomic sites showed significantly different levels of the target fatty acid. The DH lines with only Locus E-1 and the DH lines with only Locus E-III had a more than 2-fold difference in the C16:0 level (10.8% and 25.2%, respectively) although each of the loci had one copy of the elm-TE transgene. Similarly, the DH lines with only Locus E-VI and DH lines with only Locus E-II showed an 1.3-fold difference in the C16:0 level (28.7% and 36.7%, respectively) although both the loci had two copies.

The effect of genomic position on the expression of plant transgenes has been widely associated with variation in transgene expression level as is frequently observed among primary transformants from independent transformation events in a number of plant species (Allen et al., 2000; De Neve et al., 1999; Gendloff et al., 1990). The chromatin structure (heterochromatin vs. euchromatin), existence of endogenous regulator sequences such as enhancers, and discrepancy in the GC content between a transgene and the flanking sequences at the integration sites are believed to influence transgene expression (Allen et al., 2000; Chandler and Vaucheret, 2001; Fagard and Vaucheret, 2000). Significant differences in the level of the fatty acid targeted by the elm-TE transgene between DH

lines with the transgene at different loci suggested a positional effect on the expression and might indicate differences in the flanking sequences at the genomic sites. The similar levels of the target fatty acid of the DH lines with the cuphea-TE transgene at different loci did not exclude the possibility of the influence from the flanking sequences; instead, the flanking sequences at the loci (Locus C-II and C-III) could have a similar effect on the expression. Such an explanation may explain some conflicting reports. Positional effect was reported to influence the level of C12:0 targeted by the bay-TE transgene in *B. napus* (Voelker et al., 1996). In contrast, no significant positional effect was detected in studies with other transgenes in some plant species (Gendloff et al., 1990; Hobbs et al., 1990).

4.5.2 Effect of Copy Number

Comparisons between SP seeds and F_1 seeds, among transgenic DH lines with different numbers of the cuphea-TE transgene, as well as among lines with different numbers of the elm-TE transgene, all showed the effect of transgene copy number on the level of the target fatty acid C16:0.

Dosage effect of transgene copies has been demonstrated by comparing hemizygous and homozygous transgenic plants, where homozygotes are expected to have a doubled number of transgene copies compared to the hemizygotes in some studies in several plant species (Azhakanandam et al., 2000; Beaujean et al., 1998; Hobbs et al., 1993; Tenllado and Diaz Ruiz, 1999). For example, transgenic tobacco F_1 plants that originated from cross-fertilization between two different homozygous transformants with high-expressing inserts, showed an expression level that closely equalled the parents. Individual F_2 plants expressed the transgenes at 50%, 100%, 150% and 200% of parent values as the copy number in the whole genome increased from 1 to 4, whether the copies were allelic or non-allelic (Hobbs et al., 1993). In the present study, the higher levels of the target fatty acids in SP seeds than F_1 seeds suggested a positive effect of extra TE transgene copies on the level of the target fatty acids.

The mean level of the target fatty acid C16:0 in the cuphea-TE DH lines increased as the copy number of the TE transgene increased from one to five copies per haploid genome. Based on 83 DH

lines with one to five copies of the cuphea-TE transgene, copy number and target fatty acid level had a positive linear correlation ($r = 0.77^{**}$), indicating that copy number could account for near 60% ($R^2 = 0.593$) of the total variation in the level of the target fatty acid among the cuphea-TE DH lines. The positive linear correlation observed in this study was in agreement with some previous reports regarding the relationship between copy number and expression level with other transgenes (Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999; van der Hoeven et al., 1994). Transgenic tobacco plants showed increased expression levels as the copy number of the chloramphenicol acetyl transferase (CAT) transgene increased from 1 to 4, although plants with one copy sometimes produced more CAT than those with more copies (Gendloff et al., 1990). The remaining variation in the target fatty acid level which could not be explained by copy number of the TE transgenes might be due to plant variation within the same DH lines, since only one plant was tested for the fatty acid level for each DH line in this experiment, although there were replicates for DH lines with the same locus and copy number. Variation from plant to plant within the same TE transgenic DH lines was demonstrated in our another experiment. As well, variation in transgene expression was observed between asexually propagated transgenic tobacco plants (Bhattacharyya et al., 1994).

DH lines carrying the elm-TE transgene displayed a complex relationship between the copy number and the level of the target fatty acid C16:0. For example, DH lines with four copies at the two loci, Locus E-II and E-IV, showed an mean C16:0 level of 35.2%, which was significantly higher than 28.7% C16:0 for the lines with two copies at Locus E-IV, but not significantly different from 36.2% C16:0 for the lines with two copies at Locus E-II. This observation implied that, when the target fatty acid level was high (e.g. 36.2% C16:0 for the lines with two copies at Locus E-II), an addition of extra transgene copies could not further increase the target fatty acid level. When the target fatty acid level was relatively low (e.g. 28.7% C16:0 for the lines with two copies at Locus E-IV), the target fatty acid level could be increased by increasing transgene copies or by expression of the transgene at a different locus (e.g. Locus E-II). Thus, there appeared to be an upper limit, ca. 36.2% C16:0 in this study and 33% C16:0 in the primary transformants (Voelker et al., 1997), for the level of C16:0 in transgenic lines with the elm-TE transgene.

Studies with *B. napus* transgenic plants carrying the bay-TE showed that the activity of the enzyme had a linear positive correlation with the level of C12:0 targeted by the enzyme until the level reached 40% (Voelker et al., 1996). Above 40%, the linear relationship was lost and the C12:0 level increased at a lower and decreasing rate as the enzyme activity increased, with an upper limit of ca. 60% C12:0 in bay-TE transgenic lines (Voelker et al., 1996). Other enzymes, e.g. β-ketoacyl ACP synthase (KAS), which catalyzes the elongation of fatty acyl chain-ACP and acyl-ACP acyltransferase (LPAAT) which assembles fatty acids into triacylglycerols, have been proposed to be limiting factors for a further increase in the level of the target fatty acids in TE transgenic plants (Eccleston and Ohlrogge, 1998; Hawkins and Kridl, 1998; Voelker et a l., 1997; Wiberg et al., 1997). The positive linear correlation observed in this study between copy number of the cuphea-TE transgene and the level of the target fatty acid C16:0 could represent a similar relationship between the enzyme activity and the fatty acid level as was observed with the bay-TE transgenic lines. All the DH lines with the cuphea-TE transgene had less than 40% C16:0 in this study; thus, the activity of the cuphea-TE enzyme was a limiting factor. Increase in the enzyme activity by expression of more copies could result in higher levels of C16:0. However, for the elm-TE transgenic lines, the upper limit of the target fatty acid was lower than that of the bay-TE and the cuphea-TE transgenic lines. Expression of the elm-TE transgene at an appropriate genomic position with a limited number of transgene copies (e.g. at Locus E-II with two copies) produced sufficient C16:0 to reach the limiting level so that additional elm-TE transgene copies could not significantly increase the target fatty acid level. This indicated that the effect of transgene copy number is dependent on the genomic position of the copies.

CHAPTER 5

STABILITY OF THE EXPRESSION OF ACYL-ACP THIOESTERASE TRANSGENES IN *BRASSICA NAPUS* DOUBLED HAPLOID LINES

5.1 ABSTRACT

Stability of the expression of four acyl-acyl carrier protein (ACP) thioesterase (TE) transgenes was assessed in *Brassica napus* doubled haploid (DH) lines. Four transgenic parental lines, transformed individually with the bay-TE (*Uc FatB1*), elm-TE (*Ua FatB1*), nutmeg-TE (*Mf FatB1*) and cuphea-TE (*Ch FatB1*) genes, were crossed with non-transgenic cultivars, and the F_1 hybrid plants were used as microspore donors for DH line development. More than 300 DH lines, developed from microspore-derived embryos which had undergone selection for the selectable marker neomycin phosphotransferase II (npt II) with hybrid plants carrying the bay-TE or the cuphea-TE transgene as the microspore donors, were examined for the expression of the TE transgenes. Only 17 plants did not show accumulation of lauric acid (C12:0) or enhanced level of palmitic acid (C16:0), the target fatty acids of the bay-TE and the cuphea-TE transgene, respectively, in the seed oil. Polymerase chain reaction (PCR) and Southern blotting analyses showed that the lack of the accumulation of the target fatty acid was due to the escape of embryos from the kanamycin selection or existence of an incomplete T-DNA copy without the cuphea-TE transgene. No DH plant with a completely silenced TE transgene was detected. Expression of the elm-TE and the nutmeg-TE transgenes in DH_3 and DH_4 (seed generation), and the bay-TE and the cuphea-TE transgenes in DH_3, DH_4 and DH_5, were analyzed for the stability of the expression over generations. Thirty of 34 transgenic DH lines showed a stable level of the target fatty acid, C12:0 for the bay-TE and C16:0 for the other three TE, over the generations. However, there was a wide variation in the level of the target fatty acids from plant to plant within the same DH lines, with the coefficient of variation (C.V.) ranging from 4% to

16% for eight DH lines carrying the bay-TE or the cuphea-TE transgenes. This indicated the influence of developmental and experimental error on the observed level of the target fatty acids. In addition, influence of growing conditions of DH lines on the level of the target fatty acids was observed. Transgenic plants carrying the elm-TE or the cuphea-TE transgenes grown under high temperature conditions (25/20 °C, day/night) during seed development produced significantly higher levels of the target fatty acids in the seed oil compared to the plants grown under lower temperature conditions (20/15 °C) carrying the same TE transgenes.

Abbreviations: C12:0, lauric acid; C16:0, palmitic acid; DH, doubled haploid; MT, microspore treatment; npt II, neomycin phosphotransferase II; PCR, polymerase chain reaction; RT, root treatment; TE, acyl-acyl carrier protein (ACP) thioesterase.

5.2 INTRODUCTION

Conventional canola cultivars (*Brassica napus* L.) have traces of lauric acid (C12:0) and ca. 4% palmitic acid (C16:0) in the fatty acid composition of the seed oil. *Agrobacterium*-mediated transformations of *B. napus* plants with acyl-acyl carrier protein (ACP) thioesterase (TE) genes cloned from several plant species have led to the development of transgenic lines accumulating C12:0 or enhanced levels of C16:0 in the seed oil (Jones, et al., 1995; Voelker et al., 1996, 1997). Oils rich in such fatty acids have a number of food and non-food uses, e.g. manufacturing of laundry detergent, shampoo, margarine and shortening.

For practical applications, it is important that transgenes are inherited and expressed in a predictable, consistent, and stable manner (Campbell et al., 2000; Conner and Christey, 1994; Conner et al., 1998). Voelker et al. (1996) reported that *B. napus* lines with multiple copies (5-15) of the bay-TE transgene (*Uc FatB1*) were stable for more than five generations, without any apparent genetic instability and loss of transgenic phenotype (Voelker et al., 1996). However, instability in the expression of other transgenes has been observed in some studies (Assaad et al., 1993; Scheid et al.,

1991; Zhong et al., 1999). For example, in a population of *Arabidopsis thaliana* plants transgenic for a hygromycin resistance gene (*hpt*), 50% of the plants failed to transmit the resistant trait to the progeny although the complete transgene was detected in all the plants (Scheid et al., 1991).

Unstable expression of transgenes has been associated with gene silencing as gene silencing is often a reversible process (Charrier et al., 2000; Meyer and Saedler, 1996; Scott et al., 1998). Gene silencing is defined as somatically or meiotically heritable repression of gene expression that is potentially reversible and is not due to mutation (Kaeppler et al., 2000). Silencing could result from the blocking of transcription initiation, transcriptional gene silencing (TGS), or from the degradation of mRNA after transcription, post-transcriptional gene silencing (PTGS) (Chandler and Vaucheret, 2001; Matzke and Matzke, 1998; Wassenegger, 2000). Possible factors inducing gene silencing include multiple transgene copies, special structure of transgene inserts (e.g. tandem repeats and truncated copies), vector sequence, special genomic site, as well as in vitro tissue culture and growing condition of transgenic plants (Charrier et al., 2000; Dale et al., 1998; Maqbool and Christou, 1999).

However, no report was found about the stability of the expression of TE transgenes in different plant growing conditions and in doubled haploid (DH) lines which were developed through in vitro culture. Also, there is little information about the stability of the TE transgenes over generations. This study provided data regarding the stability of expression of four different acyl-ACP TE transgenes in *B. napus* DH plants.

5.3 MATERIALS AND METHODS

5.3.1 Parental Genotypes

The original seeds of transgenic parental lines TL1, TL3, TL5 and TL6, were kindly provided by Calgene Inc. (USA). TL1 was developed by transformation with the bay-TE gene *Ua FatB1* (Voelker et al., 1992, 1996), TL3 with the elm-TE *Uc FatB1*, TL5 with the nutmeg-TE *Mf FatB1*

(Voelker, 1997), and TL6 with the cuphea-TE *Ch FatB1* (Jones et al., 1995). Transgenic plants carrying the bay-TE gene showed accumulation of C12:0 in the seed oil, whereas plants carrying any of the other three TE genes accumulated enhanced levels of C16:0 in the seed oil, compared to non-transgenic *B. napus* cultivars (ca. 4% C16:0). The selectable marker for the transformations of the four TE transgenes was neomycin phosphotransferase II (npt II), the gene conferring kanamycin resistance. The three non-transgenic cultivars used as parents in this study included Apollo (Scarth et al., 1995a), AC Excel (Rakow, 1993) and Mercury (Scarth et al., 1995b).

5.3.2 DH Line Development

DH lines were developed from F_1 hybrid plants through microspore culture and chromosome doubling based on the procedure described by Ferrie and Keller (1995). The F_1's originated from crosses between the four transgenic parental lines and the three non-transgenic cultivars. Microspores were isolated from unopened flower buds of hybrid plants. Embryogenesis was induced by culturing the microspores in induction media at 32.5 °C for 3 d. When small embryos turned green in color, they were transferred to solid B5 media for regeneration.

A total of 333 DH plants were developed from embryos having undergone selection for the selectable marker npt II. For the selection, small embryos were cultured on B5 media containing 50 mg/l kanamycin for three weeks. Embryos that survived kanamycin selection remained green and were transferred to fresh B5 media without kanamycin for subsequent regeneration.

Doubling of the chromosome number was conducted using two alternative treatments: microspore treatment (MT) or root treatment (RT). For MT, microspores were cultured in induction media containing 10 mg/l colchicine in the first 48 h of the culture process. For RT, the roots of haploid plantlets were immersed in 0.34% colchicine solution for 2 h after the plantlets had been grown in soil for 3 - 4 weeks. DH_1 plants, directly from the chromosome doubling treatments (Stringam et al., 1995), were grown in a greenhouse (GH) to produce DH_2 seeds. Two more generations of propagation in the GH was conducted for production of DH_3 and DH_4 seeds. The DH_2, DH_3 and DH_4 seeds were used to grow the trials for this study as described below.

5.3.3 Trials in Growth Room and Greenhouse

Four experiments were carried out under growth room (GR) and greenhouse (GH) conditions in this study. No field experiment was conducted as the transgenic lines were not assessed for authorization of confined field trials in Canada. In each of the four experiments, individual plants were grown separately in 15-cm pots; self-pollinated seeds of each plant were harvested and tested for the fatty acid composition of the seed oil.

The objective of experiment 1 was to assess the stability of transgenic DH lines over generations. A total of 34 DH lines carrying the bay-TE, elm-TE, nutmeg-TE or cuphea-TE transgenes were tested for the stability of the target fatty acids in concurrently grown plants of two or three consecutive generations of the same transgenic lines. For the DH lines carrying the bay-TE or the cuphea-TE transgenes, two generations (DH_3 and DH_4 seeds) were tested; for the DH lines carrying the elm-TE or the nutmeg-TE transgenes, three generations (DH_3, DH_4 and DH_5) were tested. Three plants were grown for each generation of each line in a randomized complete block (RCB) design with three replicates in a GR which had controlled growing conditions: a 16-h photoperiod, 580 μE m^{-2} s^{-1} light intensity, and day/nigh temperatures of 20/15 °C.

The objective of experiment 2 was to determine the effect of growing environment on the expression of the TE transgenes. For this purpose, DH_3 plants of 42 DH lines carrying the bay-TE, elm-TE, nutmeg-TE or cuphea-TE transgenes were grown in the GR with controlled growiong conditions as described above; as well, the same lines were grown in a GH. In both the GR and the GH, the plants were grown in a completely randomized design, with one plant per line.

The objective of experiment 3 was to assess the effect of elevated temperatures during seed development on the expression of the TE. Six plants (DH_3 plants) were grown to the bolting stage in the GR for each of 10 DH lines carrying the bay-TE, elm-TE, nutmeg-TE or cuphea-TE transgenes. At the onset of flowering, three plants of each line were transferred to a GR with controlled environmental conditions as described above but with day/night temperatures of 25/20 °C until seed development was completed; the other three plants of each line remained in the GR

with 20/15 °C until seed development was completed.

The objective of experiment 4 was to test the variation within transgenic DH lines. DH_3 plants of eight DH lines, including four lines for which the chromosome number was doubled by RT and four lines by MT, were grown in the GR with 20/15 °C, in a completely randomized design. Around 12 plants were harvested for each of these lines on average.

5.3.4 Plant DNA Isolation and Characterization of DH Lines by PCR

Plant genomic DNA for PCR and Southern blotting analyses was extracted from ca. 3-5 g of cotyledon and young leaves using the CTAB (cetyltrimethylammonium bromide) procedure (Kidwell and Osborn, 1992). Extracted DNA was purified with 25:24:1 of chloroform, phenol and isoamyl alcohol (Ausubel et al., 1995).

DH lines developed from embryos that had undergone the kanamycin selection but did not show the expected fatty acid composition were characterized by PCR (Floolad et al., 1994). The sequences of the left and the right primers for amplification of an internal fragment of each of the transgenes by PCR were as follows: the primers used for an 1.0-kb fragment of the bay-TE transgene *Uc FatB1* being 5'-GAGCTTGAAAAGGTTGCCTG-3' and 5'-GGTTCTGCGGGTATCACACT-3'; the primers for a 1.1-kb fragment of the cuphea-TE being 5'- GAACTTTTATCAACCA-3' and 5'-ACCTGCCCTTCACTCAG-3'; the primers for a 0.7-kb fragment of the kanamycin resistance gene npt II being 5'-AGACAATCGGCTGCTCTGAT-3' and 5'-CTCGTCCTGCAGTTCATTCA-3'. In addition to the primers corresponding to the TE transgene, primers P1/P2, designed based on the napin gene sequence published by Kridl et al. (1991), were added in each PCR reaction, which result in a 0.5-kb internal control band for both transgenic and non-transgenic plants.

5.3.5 Preparation of DNA Probes

The probes were prepared with a non-radioactive digoxygenin (DIG)-labeling system with a PCR DIG Probe Synthesis kit (Roche, Germany). To prepare the cuphea-TE probe, the TE transgene was amplified from genomic DNA of the transgenic line with the left primer PT1, 5'-

ATTAGAGCCTCGGCTTCACTC-3' and the right primer PT2, 5'-GGATCCCATTGGATGATCTTT -3' by PCR. The amplified DNA fragment was cloned with the pGEM®-T Easy Vector and JM109 Competent Cells (Promega, Madison, USA). A positive white colony was grown overnight in LB Broth media and the plasmid DNA carrying the cuphea-TE gene was extracted (Ausubel et al., 1995). With the plasmid DNA having the TE gene as the template, an 1.1-kb probe for the TE was produced and DIG-labeled by PCR under the presence of DIG-dUTP. A 0.7-kb DIG-labeled probe for the kanamycin resistance gene npt II was also prepared with plasmid DNA containing the npt II gene as the template.

5.3.6 Southern Blotting Analysis

Genomic DNA (5-10 ug) of cuphea-TE transgenic plants was digested with *Nsi*I, separated by electrophoresis on 0.8% agarose gel, and blotted onto a nylon hybridization transfer membrane (Hybond-N+, Amersham). *Nsi*I has an unique site, located in the napin promoter, in the T-DNA region (Jones et al., 1995). The subsequent procedure was conducted with a DIG non-radioactive hybridization and chemiluminescent detection system (Roche, Germany). Hybridization took place overnight in a hybridization buffer, DIG Easy Hyb solution, at 50 °C under the presence of the 1.1-kb cuphea-TE probe, with stringency washes at 68 °C in 2 x Wash solution (2 x SSC, containing 0.1 SDS) for 2 x 30 min. After washes and blocking, the membrane was incubated with a dilution of Anti-Digoxigein-Fab fragment conjugated to alkaline phosphatase (AP). For production of luminescent signal, an 1:100 dilution of the AP substrate CDP-Star was scattered on the membrane. The signal was recorded with standard X-ray films. Re-probing of the membrane with a second probe, the DIG-labeled 0.7-kb npt II fragment, was conducted with the same procedure as described above after removing the chemiluminescent substrate CPD-Star with H_2O and stripping the TE probe with an alkaline probe-striping solution (0.2 NaOH, 01.% SDS). The sizes of the bands on the films were estimated based on the 1 KB PLUS DNA Ladder (GibcoBRL, Canada) running alongside the digested DNA samples.

5.3.7 Determination of the Fatty Acid Composition

The fatty acid composition of seed oils was determined by gas chromatography of the methyl ester

derivatives of the fatty acids (Hougen and Bodo, 1973; Knutzon et al., 1992). A sample of 10 seed was picked randomly from the seeds of each plant to be tested, the seed oil was extracted overnight with 1 ml heptane. 300 ul of 0.5 M sodium methoxide was added for methyl ester derivitization. The oven temperature was programmed to go from 190 to 230 °C. The level of a fatty acid is reported as a percentage of the total fatty acids (Chen and Beversdorf, 1990; Knutzon et al., 1992; Pleines and Friedt, 1989; Sommerville, 1993).

5.3.8 Statistical Analysis

Multiple comparisons and correlation studies were performed as described by Ott (1993).

5.4 RESULTS

5.4.1 Stability of DH Plants Developed from Embryos Selected with Kanamycin

A total of 333 DH plants were developed from embryos which had undergone kanamycin selection, and the DH plants were tested for the fatty acid composition of the seed oil (Table 5.1).

Since the embryos survived kanamycin selection, it was expected that the DH plants developed from the embryos would have the kanamycin resistance gene npt II and the TE transgene, thus accumulating the target fatty acid of the TE in the seed oil. Based on the fatty acid composition of the seed oil, however, some of the DH plants did not show the expected transgenic phenotype (Table 5.1). Of the 15 DH plants that originated from crosses between the bay-TE transgenic parental line TL1 and non-transgenic plants, three plants showed the same phenotype as the non-transgenic control plants with no accumulation of C12:0. Among 318 DH plants originating from the crosses between the cuphea-TE transgenic parental line TL6 and non-transgenic plants, 17 plants had C16:0 levels ranging from 3.3% to 6.8%, the same phenotypes as the non-transgenic control plants, indicating no expression of the cuphea-TE transgene.

A possible cause for the lack of the expected transgenic genotype in these DH plants having undergone selection was that the TE transgenes in these plants were silenced. The other possibilities

Table 5.1 Number of DH plants developed from microspore-derived embryos having undergone kanamycin selection which were produced by microspore culture from crosses of transgenic parental lines TL1 and TL6 with non-transgenic plants with the minimum and maximum levels of the target fatty acids in the seed oil of the DH plants.

		Phenotypic class†		Non-transgenic
Cross‡	Parameter	Transgenic	Non-transgenic	Control plant
TL1-crosses:				
	No. of plants	12	3	5
	C12:0 level:			
	min., %	6.9	-§	-
	max., %	38.1	-	-
TL6-crosses:				
	No. of plants	301	17	5
	C16:0 level:			
	min., %	13.3	3.3	3.9
	max., %	42	6.8	5.0

† The DH plants were divided into transgenic and non-transgenic classes based on the level of the target fatty acid, C12:0 for the plants from the TL1-crosses and C16:0 for plants from the TL6-crosses. The plants having showed the accumulation of 6.9% or more C12:0 or 13.3% or more C16:0 were included in the transgenic classes.

‡ The crosses of TL1 and TL6 with non-transgenic cultivars Apollo, AC Excel or Mercury.

§ Less than 1% C12:0.

were that these plants might have originated from non-transgenic embryos that escaped from the kanamycin selection, or the plants might carry incomplete T-DNA copies which contained only the npt II but not the TE transgene. In order to distinguish between these possibilities, PCR and Southern blotting analyses were performed.

In PCR analyses the three DH plants, represented by lanes 1-3 (Fig. 5.1), from crosses between the bay-TE transgenic parent TL1 and non-transgenic parents, exhibited only an internal control band identical to that of the non-transgenic breeding line 212/86 (lane 5). In PCR analyses with primers for amplification of an 1.0-kb internal fragment of the bay-TE transgene, the transgenic parent TL1 showed the expected 1.0-kb band (lane 4, Fig. 5.1a), but the three lines did not. Similarly, in PCR analyses with primers for a 0.7-kb fragment of the npt II gene, the three plants did not show any band at ca. 0.7 kb (Fig. 5.1b). Therefore, the DH lines did not carry either the bay-TE transgene or

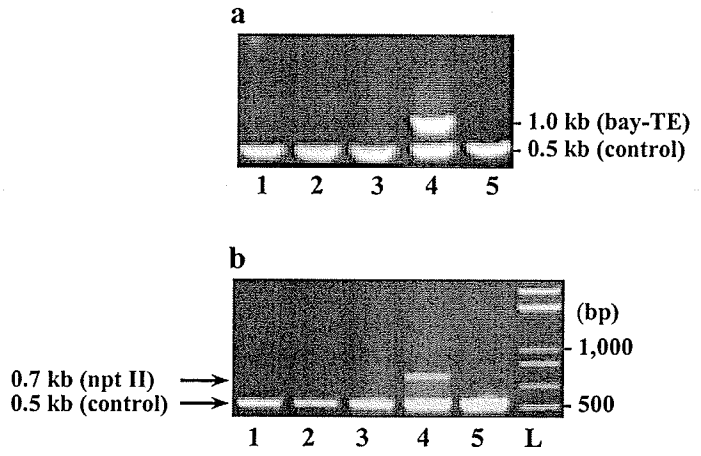


Figure 5.1 PCR analyses of the three DH Lines without accumulation of C12:0 in the seed oil developed from embryos selected with kanamycin. a: PCR with the bay-TE gene primers for an 1.0-kb fragment and the napin promoter primers for a 0.5-kb control band; b: PCR with the npt II gene primers for a 0.7-kb fragment and the napin promoter primers. Lanes 1- 3, the three DH lines; lane 4, the bay-TE transgenic parental line TL1; lane 5, non-transgenic control. Sizes of DNA molecular weight markers (L) are indicated in base pair (bp).

the kanamycin resistance selectable marker. These three lines must have originated from non-transgenic embryos which escaped from the kanamycin selection, thus the lack of C12:0 accumulation was not due to TE transgene silencing.

Of the 17 DH plants developed from crosses between the cuphea-TE transgenic parental line TL6 and non-transgenic parents, eight of them did not carry the npt II gene (lanes 2, 6, 7, 8, 10, 11, 14, 16 in Fig. 5.2), indicating that these lines escaped from the kanamycin selection. The remaining nine lines showed the 0.7-kb band representing the npt II gene (lanes 1, 3, 4, 5, 9, 12, 13, 15, 17).

However, in PCR analyses with primers for amplification of an 1.1-kb fragment of the cuphea-TE transgene, these nine lines did not show the 1.1-kb band (lanes 1 - 17, Fig. 5.3) seen in the transgenic control (lane 18, Fig. 5.3), indicating that the T-DNA in these plants was incomplete, which contained the npt II gene but not the cuphea-TE gene. Thus, the lack of the transgenic phenotype in these plants was not due to gene silencing, but rather to the absence of the cuphea-TE transgene.

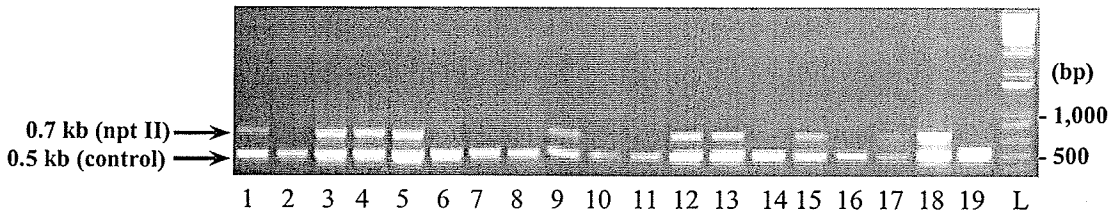


Figure 5.2 PCR analyses using the npt II gene primers for a 0.7-kb fragment and the napin promoter primers for a 0.5-kb control band of the 17 DH lines developed from embryos selected without kanamycin without accumulation of enhanced levels of C16:0 in the seed oil. Lanes 1-17, the DH lines; lane 18, the cuphea-TE transgenic parent; lane 19, non-transgenic control. Sizes of DNA molecular weight markers (L) are indicated in base pairs (bp).

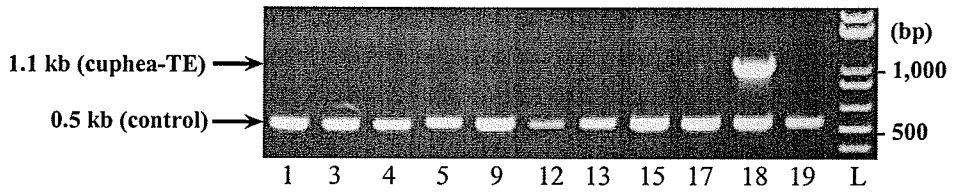


Figure 5.3 PCR analyses using the cuphea-TE primers for amplification of an 1.1-kb fragment and the napin promoter primers for a 0.5-kb control band of the nine DH lines which showed the npt II gene. Lanes 1-17, the nine DH lines with the same lane no. as in Fig. 5.2; lane 18, the cuphea-TE transgenic parent TL6; lane 19, non-transgenic control. Sizes of DNA molecular weight markers (L) are indicated in base pair (bp).

The possible origin of the incomplete T-DNA copies was investigated by Southern blotting analyses. In the Southern blotting analyses with an internal fragment of the cuphea-TE gene as the probe, the transgenic parental line TL6 displayed five bands, which were approximately 11.5, 9.0, 8.5, 8.0 and 4.0 kb long, respectively (Fig. 5.4a). By re-probing of the same membrane with an internal fragment of the npt II gene as the probe, TL6 also showed five bands, with four of the bands having the same sizes as detected with the TE gene probe, i.e. the bands of 11.5, 9.0, 8.5 and 8.0 kb long, respectively (Fig. 5.4b). This indicated that the T-DNA copies represented by the four bands in the parental line had both the npt II and the TE transgenes (referred to as compete T-DNA copies). Another band detected with the npt II gene probe was 6.0 kb long (Fig. 5.4b), whereas no band of such a length was detected with the TE probe (Fig. 5.4a), indicating that the T-DNA copy represented by the 6.0-kb

band contained the npt II gene but not the TE transgene. The 4.0-kb band detected with the TE probe suggested an incomplete T-DNA copy having the TE but not the npt II gene since no band of 4.0-kb in length was detected with the npt II probe.

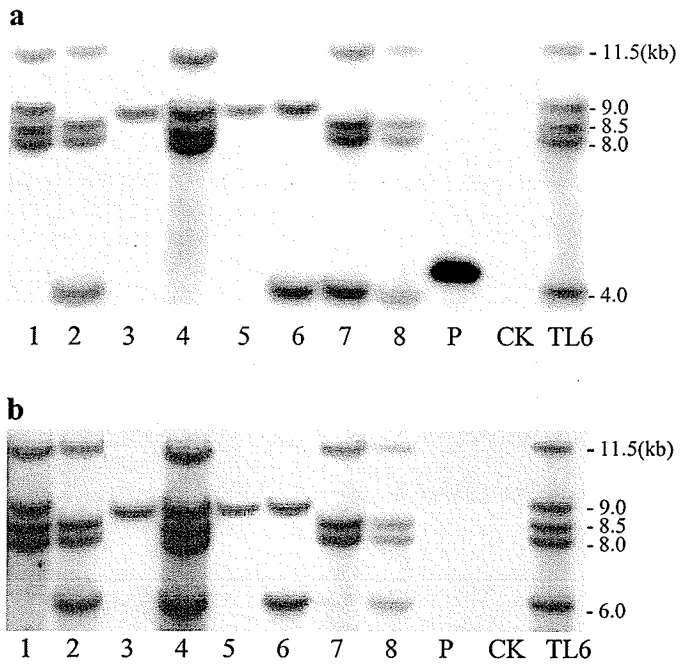


Figure 5.4 Southern blotting analyses of cuphea-TE DH lines by probing with the cuphea-TE transgene probe (a) followed by re-probing with the npt II gene probe (b). P, a plasmid carrying the cuphea-TE gene; CK, non-transgenic control plants. The sizes of the bands, estimated based on the phage lambda *Hind* III 1-kb ladder, are shown in kilobase (kb).

Southern blotting of DH plants provided confirmation that the copies represented by the four larger bands had both the npt II and the TE transgenes, because the four bands could be detected by both the npt II and the cuphea-TE probes (lanes 1-8). Also, the results from the DH plants confirmed that the 4.0-kb band detected with the TE probe represented an incomplete T-DNA copy without the npt II transgene, and the 6.0-kb band detected with the npt II probe represented another incomplete T-DNA copy without the cuphea-TE gene. In addition, the incomplete T-DNA copy with the npt II but no cuphea-TE transgene (represented by the 6.0-kb band) segregated from the complete T-DNA copies, and from the incomplete copy with TE but not the npt II gene (represented by the 4.0-kb band). For example, some plants (lanes 1, 3, 5 and 7, Fig. 5.4b) showed some or all the four bands

representing the complete T-DNA copies but not the 6.0-kb band, and other plants (lanes 4 and 7, Fig 5.4a, b) showed only the 6.0- or the 4.0-kb but not the both. Therefore, it was possible that some DH plants developed from the hybrids between TL6 and non-transgenic plants inherited only the incomplete T-DNA that had the npt II gene but not the cuphea-TE gene.

5.4.2 Stability of DH Lines Carrying TE Transgenes over Generations (Experiment 1)

Bay-TE transgenic DH lines

Most of DH lines carrying the bay-TE transgene showed stability in the level of target fatty acid (C12:0) over the two generations, DH_3 and DH_4 seeds (Table 5.2). DH_3 seeds of the 11 bay-TE lines had an overall mean C12:0 levels of 32.0%; the DH_4 seeds harvested from concurrently grown plants of the same lines had an overall mean C12:0 level of 30.4%. These values are not significantly different. Of the 11 lines, each of eight lines showed no significant difference in the C12:0 level between the two generations. For the other three lines, i.e. lines 436 and 570 from TL1 x AC Excel and line 962 from TL1 x Apollo, the C12:0 levels in the DH_3 and DH_4 seeds were significantly different, which could be caused by a plant to plant variation. For example, DH_3

Table 5.2 Mean lauric acid (C12:0) level (%) in the seed oil of DH_3 and DH_4 seeds of 11 bay-TE transgenic lines developed from crosses of the bay-TE transgenic parental line TL1 with three non-transgenic cultivars Apollo, AC Excel, or Mercury.

Cross:	TL1 x Apollo				TL1 x AC Excel				TL1 x Mercury			Overall
Line:	532	556	962	870	436	464	570	452	995	1083	1345	mean
DH3	40.1a†	30.8a	38.1a	36.6a	36.8a	41.1a	39.1a	22.0a	21.6a	26.6a	19.0a	32.0a
DH4	42.3a	32.5a	31.6b	38.2a	30.3b	42.1a	31.5b	22.5a	19.5a	25.4a	19.0a	30.4a

† The means followed by the same letter in the same column were not significantly different by Fisher's LSD test at the 0.05 level.

seeds of one plant of line 436 showed a much higher C12:0 level (41.7%) than the other two plants

(32.3% and 36.6%, respectively). Because all the plants of the two generations of these three lines accumulated high levels of the target fatty acid of the bay TE transgenes (over 26%), the differences between the generations were not caused by transgene silencing.

Elm-TE and Nutmeg-TE Transgenic DH Lines

Stability in the level of C16:0 targeted by the elm-TE and nutmeg-TE transgenes was assessed over three generations, DH_3, DH_4 and DH_5 seeds (Table 5.3). All the six elm-TE transgenic lines

Table 5.3 Mean palmitic acid (C16:0) level (%) in the seed oil of DH_3,DH_4 and DH_5 seeds of six elm-TE transgenic DH lines developed from crosses of the elm-TE transgenic parental line TL3 and the three non-transgenic cultivars Apollo, AC Excel, or Mercury.

Cross:	TL3 x Apollo		TL3 x AC Excel		TL3 x Mercury		Overall
Line:	256	276	250	255	262	351	mean
DH_3	19.4a†	22.0a	24.7a	15.5a	22.1a	17.6a	20.2a
DH_4	21.5a	21.5a	27.00a	18.1a	22.7a	16.8a	21.3a
DH_5	21.4a	21.2a	24.32a	17.7a	23.1a	16.0a	20.6a

† The means followed by the same letter in the same column were not significantly different by Fisher's LSD test at the 0.05 level.

from crosses with different non-transgenic parents showed stable expression over the three generations. The five nutmeg-TE lines also showed stability over the three generations. The mean C16:0 levels at DH_3, DH_4 and DH_5 were 20.9%, 22.1% and 22.1%, respectively, not significantly different (Table 5.4). Of the five lines, there was only one line (line 25) which showed a lower C16:0 level in DH_3 seed, but DH_4 and DH_5 seeds were not significantly different.

Cuphea-TE Transgenic DH Lines

The mean C16:0 levels of the DH_3 and DH_4 seeds of the 12 cuphea-TE transgenic lines were almost the same, 24.2% and 24.3%, respectively (Table 5.5). None of the cuphea-TE transgenic lines showed a significant difference between the two generations in the C16:0 level. As well, no

difference was found in the stability between the lines from crosses with different non-transgenic parents.

Table 5.4 Mean palmitic acid (C16:0) level (%) in the seed oil of DH_3, DH_4 and DH_5 seeds of five nutmeg-TE transgenic DH lines developed from crosses of the nutmeg-TE transgenic parental line TL5 and the three non-transgenic cultivars Apollo, AC Excel, or Mercury.

Cross:	TL5 x Apollo		TL5 x AC Excel		TL5 x Mercury	Overall
Line:	295	346	104	126	25	mean
DH_3	20.6a†	23.7a	20.0a	24.1a	16.1b	20.9a
DH_4	20.3a	26.8a	21.9a	22.0a	19.4a	22.1a
DH_5	21.4a	25.3a	20.8a	24.0a	18.9ab	22.1a

† The means followed by the same letter in the same column were not significantly different by Fisher's LSD test at the 0.05 level.

Table 5.5 Mean palmitic acid (C16:0) level (%) in the seed oil of DH_3 and DH_4 seeds of 12 cuphea-TE transgenic DH lines developed from crosses of cuphea-TE transgenic parental line TL6 and the three non-transgenic cultivars Apollo, AC Excel or Mercury.

Cross:	TL6 x Apollo				TL6 x AC Excel				TL6 x Mercury				Overall
Line:	514	861	1202	1332	424	485	1237	1489	448	641	725	790	mean
DH_3	20.3a†	24.0a	24.6a	25.7a	22.3a	20.9a	20.0a	21.6a	28.3a	28.5a	24.4a	29.4a	24.2a
DH_4	21.0a	23.1a	24.4a	24.6a	22.7a	19.6a	20.2a	22.0a	29.3a	27.0a	26.2a	31.2a	24.3a

† The means followed by the same letter in the same column were not significantly different by Fisher's LSD test at the 0.05 level.

5.4.3 Influence of Growing Environment on the Target Fatty Acids (Experiment 2)

The effect of growing conditions on the expression of the TE transgenes in DH lines was detected by comparing plants grown in the GR with plants of the same lines grown in the GH. Plants of the 12 bay-TE transgenic DH lines in the GH showed a mean C12:0 level of 30%, not significantly different from the mean level of 31% of the plants grown in the GR (Table 5.6).

DH plants carrying the elm-TE transgene grown in the GH, however, showed significantly higher levels of the target fatty acid C16:0 (26.2% on average) in the seed oil than plants of the same lines in the GR (21.6% on average, Table 5.6). Similarly, transgenic lines having the nutmeg-TE or the cuphea-TE transgene accumulated ca.4% more C16:0 when grown in the GH than in the GR. A possible cause for the enhanced levels of the target fatty acids in the plants grown in the GH, compared to plants in the GR, was the influence of the temperature, although the influence of other environmental factors, e.g. light intensity and photoperiod, could not be excluded.

Table 5.6 Mean level (%) of the target fatty acids in DH_4 seed oil of 42 DH lines with the bay-TE, elm-TE, nutmeg-TE or cuphea-TE transgene grown in a growth room (GR) with controlled environment and in a greenhouse (GH).

Transgene (Target fatty acid)	No. of lines	Growing location		Difference†
		GH	GR	
	no.	%		
Bay-TE (C12:0)	12	30.0	31.0	-0.9
Elm-TE (C16:0)	12	26.2	21.6	4.6 **
Nutmeg-TE(C16:0)	6	26.2	21.9	4.2 **
Cuphea-TE(C16:0)	12	29.5	24.9	4.5 **

** Significant at the 0.01 level by paired-t test.
† Difference between the mean fatty acid levels of plants grown in the GH and in the GR.

5.4.4 Influence of Temperature on the Fatty Acid Composition of TE Transgenic DH Lines (Experiment 3)

Exposure to different temperatures during seed development did not result in significantly different levels of the target fatty acids in the two bay-TE DH lines and the three nutmeg-TE DH lines (Table 5.7). The level of C12:0 in the seed oil of the DH lines carrying the bay-TE transgene grown at 25/20 °C of day/night temperatures was not significantly different from the C12:0 level of the same lines grown at lower temperatures, 20/15 °C. As well, the C16:0 level in the seed oil of the DH lines carrying the nutmeg-TE transgene grown at 25/20 °C and 20/15 °C was not significantly different. However, the seed oil of the six DH lines carrying the elm-TE or the cuphea-TE transgenes showed significantly different levels of the target fatty acid C16:0 under the two different growing

Table 5.7 Mean fatty acid composition of DH lines carrying the bay-TE, elm-TE, nutmeg-TE, or cuphea-TE transgenes grown under low temperatures (20/15 °C, day/night) and high temperatures (25/20 °C) during seed development.

Trans-gene	Tempe-rature	Para-meter†	Fatty acid								No. of line/plant§
			C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	VLCF‡	
			%								no.
Bay-TE	High	Mean	28.1	3.5	3.8	1.3	33.4	12.7	2.2	14.5	2/4
	Low	Mean	29.8	3.1	3.2	1.0	29.0	11.5	4.3	17.3	2/4
		diff.	-1.7	0.4	0.6	0.3	4.4	1.2 *	-2.1	-2.8 *	
Elm-TE	High	Mean	-¶	8.9	29.8	2.3	31.8	18.2	3.6	2.2	3/9
	Low	Mean	-	7.0	26.8	1.8	37.5	16.4	7.2	1.7	3/9
		diff.		1.9 **	3.1 **	0.5 *	-5.7 **	1.9 **	-3.6 **	0.5	
Nutmeg-TE	High	Mean	-	12.8	28.1	7.8	30.1	15.9	0.9	3.9	2/4
	Low	Mean	-	11.8	27.0	5.6	31.8	18.9	1.1	3.3	2/4
		diff.		1.0	1.0	2.2	-1.8	-3.0	-0.2 *	0.6	
Cuphea-TE	High	Mean	-	2.5	42.0	2.4	29.3	18.2	2.7	2.4	3/9
	Low	Mean	-	0.7	28.5	1.7	41.4	17.6	7.3	2.1	3/9
		diff.		1.7 **	13.4 **	0.7 **	-12.1 **	0.6	-4.6 **	0.3	

*, ** Significant at the 0.05, 0.01 levels, respectively, by Fisher's LSD test.
† "diff.", difference between the mean fatty acid levels of plants under the two temperature conditions.
‡ Very long chain fatty acids, including C20:0, C20:1, C22:0 and C22:1.
§ The number of the DH lines / the number of the plants for the DH lines.
¶ Less than 1%.

temperature conditions (Table 5.7). At 25/20 °C, the mean C16:0 level of the three elm-TE transgenic lines reached 29.8%, significantly higher than 26.8% C16:0 at 20/15 °C. For the three DH lines carrying the cuphea-TE transgene, the higher temperatures led to a mean C16:0 level of 42.0%, compared to 28.5% under the lower temperature condition.

The temperature during seed development also had an effect on the other fatty acids of the seed oil (Table 5.7). The increase in the C16:0 level due to higher temperatures during seed development was accompanied by increases in the levels of C14:0 (myristic acid) and C18:0 (stearic acid), and

decreases in the levels of C18:1(oleic acid) and C18:3 (linolenic acid) for both the elm-TE DH lines and the cuphea-TE DH lines. For example, as the C16:0 level increased from 26.8% in the seed oil of the elm-TE lines at the lower temperatures to 29.8% at the higher temperatures, the C14:0 and C18:0 levels increased from 7.0 and 1.8% to 8.9 and 2.3%, respectively (Table 5.7). One bay-TE transgenic lines developed from TL1 x Mercury produced ca. 10% C20:1, with 16-22% C22:1 depending on the temperature. No lines from crosses with Mercury as the non-transgenic parent were included in this experiment for the other three TE transgenes.

5.4.5 Variation among Plants within TE Transgenic DH Lines (Experiment 4)

The seed oil of individual plants within the same transgenic DH lines exhibited variation in the level of the target fatty acids as shown by the scatter plot of the C12:0 and C16:0 levels in the seed oil of individual plants (Fig. 5.5). The range of the C12:0 level in the seed oil of individual plants of

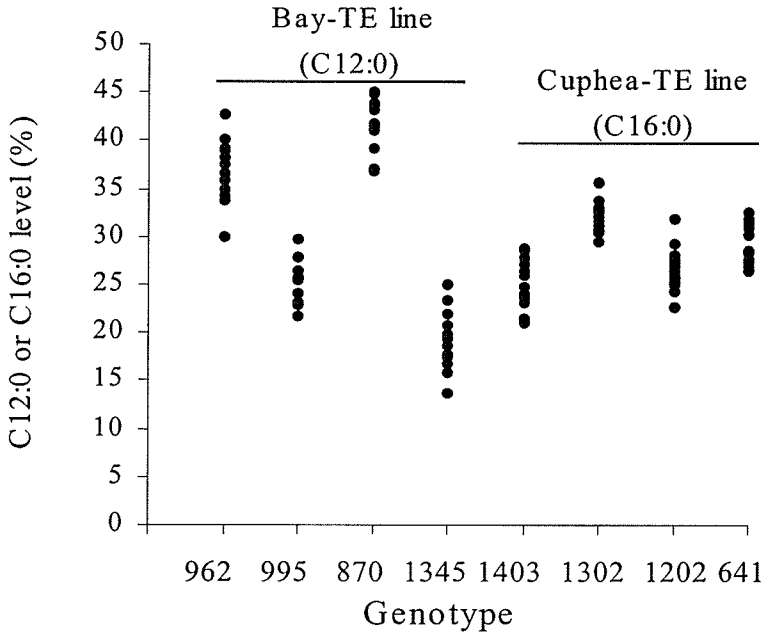


Figure 5.5 Lauric acid (C12:0) or palmitic acid (C16:0) level (%) in the seed oil of individual DH plants of eight DH lines carrying the bay-TE or the cuphea-TE transgenes. Each dot represents the mean of two GC tests of the same plant.

DH line 962, a line carrying the bay-TE transgene, was from 30.0 to 42.6%, an 1.4-fold difference between the maximum and the minimum levels. The other seven DH lines carrying the bay- or the cuphea-TE transgenes had an 1.2 to 1.8 fold difference in the level of the target fatty acids within the same lines.

The plant-to-plant variation in the level of the target fatty acids was not associated with the in vitro culture process involved in the DH line development. Coefficient of variation (C.V.), standard deviation (s) over mean in percentage, was estimated for the DH lines in order to provide comparison of the variation between the lines (Table 5.8). Because the magnitude of the standard deviation is

Table 5.8 Number of plants, mean level of lauric acid (C12:0) and palmitic acid (C16:0), standard deviation (s) and coefficient of variation (C.V.) for eight DH lines carrying the bay-TE or the cuphea-TE transgene.

	No. of	Fatty acid			Source of the line	
Genotype	plants	level (%)†	s	C.V.(%)	Cross‡	Treatment§
Bay-TE line:		C12:0:				
962	12	37.0b	3.3	8.9	A/TL1	MT
995	9	25.1c	2.4	9.6	M/TL1	MT
870	13	41.6a	2.5	6.1	A/TL1	RT
1345	13	19.1d	3.0	15.8	M/TL1	RT
Cuphea-TE line:		C16:0:				
1403	12	25.4c	2.6	10.2	A/TL6	MT
1302	13	32.1a	1.5	4.7	M/TL6	MT
1202	12	26.6c	2.3	8.6	A/TL6	RT
641	14	29.5b	2.2	7.4	M/TL6	RT

† Multiple comparison was performed between lines carrying the same TE transgenes. Means followed by the same letter were not significant at the 0.05 level.

‡ A, Apollo; M, Mercury.

§ The methods used for doubling of the chromosome number: MT, microspore treatment; RT, root treatment.

in proportion to the mean (Bowman and Watson, 1997) and the mean levels of the target fatty acid

of the DH lines showed significant differences (Table 5.8), the comparison of the variation between lines was not based on the standard deviation. The C.V. of the target fatty acids ranged from 4.7% to 15.8% in the eight DH lines. The four DH lines for which the chromosome number was doubled by MT showed similar C.V. (8.4% on average) to the four lines doubled by RT (9.5%), with some DH lines (e.g. lines 962 and 995) by MT having a C.V. value smaller than lines by RT (e.g. line 1345). RT is applied to the haploid plants after the in vitro culture process; therefore, any mutated loci caused by the culture process would be homozygous. However, if simple mutations occurred during the in vitro culture after chromosome doubling by MT, the mutated loci would be in heterozygous state. Segregation of the heterozygous loci would increase the variation among plants within the DH lines. Therefore, the similar magnitude of the variation of the DH lines from the two treatments, RT and MT, indicated that no mutation with significant influence on the target fatty acids of the TE had occurred in the in vitro culture process.

5.5 DISCUSSION

5.5.1 Stability of DH Plants from Embryos Selected with Kanamycin

The procedure of DH line development includes an in vitro culture period (Ferrie and Keller, 1995; Ilic-Grubor and Fowke, 1997). It has been reported that during in vitro culture, DNA methylation may occur (Brown et al., 1990; Kaeppler and Phillips, 1993; Olhoft 1996), which could alter chromatin structures, thus leading to variation in the expression (Kaeppler et al., 2000). Gene silencing due to in vitro culture has been observed (Kaeppler et al., 2000). In the present study, in order to determine the possibility of in vitro culture inducing silencing of TE transgenes, more than 300 DH plants developed from plants hemizygous for the bay-TE or the cuphea-TE transgenes were examined. Since the plants were regenerated from embryos that survived kanamycin selection, it was expected that the plants would have the kanamycin resistance selectable marker gene and the desired TE transgene, thus showing accumulation of C12:0 (the fatty acid targeted by the bay-TE) or an enhanced level of C16:0 (the fatty acid targeted by the cuphea-TE). Most of the plants (95%) expressed the TE transgene. The remaining plants did not show the expected transgenic phenotype

since they did not carry the TE transgene, with part of the plants being regenerated from embryos that escaped from the kanamycin selection and the others carrying incomplete T-DNA copies that had no TE transgene. No plants with a silenced TE transgene were detected. Therefore, the in vitro culture process does not necessarily induce gene silencing, influencing the level of the target fatty acids of TE transgenes. Influence of the tissue culture process on the expression of the bay-TE transgene has been excluded in a previous study (Voelker et al., 1996).

5.5.2 Stability of Expression of TE transgenes in DH lines from Generation to Generation

Stable expression over generations has been observed in studies with various transgenes (Fearing et al., 1997; McCabe et al., 1999; Scott et al., 1998), including the bay-TE transgene in *B. napus* plants (Voleker et al.,1996). The expression level of a transgenic CryIA(b) gene, for example, in *Bt* maize lines, were stable over successive backcross generations, without any significant difference among BC_1, BC_2, BC_3 and BC_4 populations planted concurrently (Fearing et al., 1997). In the present study, stability of the expression over generations was examined for four different TE transgenes by analysis of the seed oil fatty acid composition of concurrently grown transgenic DH plants of different generations. The level of the fatty acids targeted by the four TE transgenes remained stable in transgenic DH lines expressed the same TE transgene across the generations tested, two generations (DH_3 and DH_4 seeds) for the bay-TE and the cuphea-TE transgenic lines and three generations (DH_3, DH_4 and DH_5 seeds) for the elm-TE and the nutmeg-TE transgenic lines.

5.5.3 Effect of Growth Condition on the Expression of TE Transgene

Growth conditions were often shown to influence the expression of transgenes (Matzke et al., 1994; McCabe et al., 1999; Senior, 1998). Increased temperature (Conner et al., 1998; Köhne et al., 1998; Matzke et al., 1994) and high light intensity (van der Krol et al., 1990) were reported to affect transgene expression. For example, kanamycin-sensitive progeny from self-pollination of homologous 1-locus tobacco transgenic lines occurred at a frequency of $0.5\text{-}5.9 \times 10^{-5}$ under close-to-optimum environmental conditions, but the frequency became as high as $1.5\text{-}3.8 \times 10^{-3}$ under heat and/or drought stress (Conner et al., 1998). As well in tobacco, different heat stability of different sequences was reported, where one sequence showed reduced expression but the expression of a

GC-rich sequence coding for the same enzyme was stable under the same heat stress (Köhne et al., 1998). Similarly, influence of growing conditions on the expression of three of the four TE transgenes, the elm-TE, nutmeg-TE and cuphea-TE transgenes, was detected in this study.

The seed oil of DH lines carrying the elm-TE or the cuphea-TE transgenes showed higher levels of the target fatty acid C16:0 under higher temperatures (25/20 °C, day/night) compared to lower temperatures (20/15 °C). Influence of temperature on the fatty acid composition of the seed oil of non-transgenic *B. napus* cultivars has been reported (Deng and Scarth, 1998; Wilmer et al., 1996; Pritchard et al., 2000). However, the mechanism underlying the response of the TE transgenic plants to the temperature were different from that of non-transgenic *B. napus* cultivars. For example, increased temperature led to enhanced levels of saturated fatty acids (C16:0 and C18:0) and C18:1 in the seed oil of non-transgenic cultivars (Deng and Scarth, 1998), whereas for the TE transgenic lines the high temperature condition resulted in an increase in the level of the target fatty acid C16:0 accompanied by a decrease in the C18:1 level. It is possible that higher temperatures increased the relative activity of the TE compared to the enzymes for the synthesis of the C18 fatty acids, e.g. KAS II, an enzyme responsible for the elongation of C16:0 to C18:0, thus facilitating accumulation of the target fatty acids of the TE with a reduction in the percentage of C18:1.

5.5.4 Variation within the Same Transgenic DH Lines in the Expression of TE Transgenes

Individual plants of the same DH lines are expected to be genetically identical (Wenzel and Foroughi-Wehr, 1994). However, variation in the level of the target fatty acids was observed between plants within each of the eight lines carrying the bay-TE or the cuphea-TE transgenes. The coefficients of variation (C.V.) of the eight lines were from 5% to 16%. Similarly, variation in the expression level of a GUS transgene was observed in asexually propagated transgenic tobacco plants, which had C.V.values ranging from 4% to 20% (Bhattacharyya et al., 1994). Such plant-to-plant variation among genetically identical individuals has been ascribed to environmental and experimental error (Bhattacharyya et al., 1994).

Variation induced by in vitro culture has been reported (Evans, 1989; Kaeppler et al., 2000).

However, in the present study, the TE transgenic DH lines for which chromosome number was doubled by MT had similar variation to the lines doubled by RT in the level of the target fatty acids, indicating that the in vitro culture process did not increase the plant-to-plant variation, or did not affect the homogeneity, in the target fatty acids of the TE transgenes. These results were consistent with studies on the genetic stability of DH progenies in cereals, in which about 90% of the DH lines were estimated to be genetically uniform (Hu and Kasha, 1997).

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

The effects of (nuclear) recipient genotype, cytoplasm, maternal plant, genomic position and copy number, as well as the stability of the expression over generations and in different growing conditions, were assessed by analysis of the fatty acid composition of transgenic F_1 seeds and DH lines from crosses of four TE transgenic parents with non-transgenic cultivars of distinct fatty acid compositions in the seed oil. The study has provided valuable guidance for the selection of parental genotypes, offspring and growing environments in breeding programs and commercial production of TE transgenic cultivars.

6.1 Effect of Recipient Genotypes on the Expression of the TE Transgenes

Comparison of the fatty acid compositions of TE transgenic *B. napus* plants with the plant species from which the TE genes were cloned has suggested that the recipient genotype could influence the specificities of TE for the target fatty acids (Davies, 1993; Dehesh et al., 1996; Jones et al., 1995; Voelker et al., 1997). For example, *C. hookeriana* accumulates twice as much C8:0 as C10:0 in the seed oil and does not accumulate a high amount of fatty acids with a chain length longer than C10:0; but *B. napus* plants transformed with the TE gene from this species produced more C10:0 than C8:0, and high level of C16:0 (Jones et al., 1995). Such differences in the relative levels of the target fatty acids between the transgenic plants and the original species have also been found by other researchers (Davies, 1993; Dehesh et al., 1996; Voelker et al., 1997). A possible explanation is that other enzymes in the fatty acid biosynthetic pathway, e.g. KAS, differ between the recipient genotypes and can influence the relative levels of the target fatty acids (Voelker et al., 1997).

However, in the present study, when the TE transgenes were expressed in different *B. napus* recipient genotypes, the relative levels of the target fatty acids of the same TE were similar. For example, bay-TE transgenic lines always accumulated a much higher level of C12:0 than C14:0, the two target fatty acids of the bay-TE, regardless of the difference in the non-transgenic parent and in

the C22:1 level between the DH lines. For the elm-TE, nutmeg-TE and cuphea-TE, C16:0 was the major target fatty acid. The relative levels of the target fatty acids of the TE observed in this study were consistent with those observed in the original transformants of the TE (Jones et al., 1995; Voelker et al., 1996, 1997). These results imply that, within *B. napus,* compared to other plant species, there is a greater similarity in the enzymes, such as KAS, that may influence the specificity of TE for the target fatty acids.

There is no report about the influence of recipient genotypes on the level of the major target fatty acids of the TE. Some studies with other types of transgenes have shown the effect of recipient genotype on transgene expression (Blundy et al., 1991; Scott et al., 1998; Xu et al., 1997). For example, a study with a white clover line (*Trifolium repens* L.) carrying an intact copy of the GUS gene showed a four-fold variation in the GUS activity between plants, even though each plant contained one copy of the same T-DNA insert. The progeny from plants with a high level of expression showed a higher GUS activity in the next generation than those from plants with a low expression, suggesting the influence of genetic factors in the recipient genotypes (Scott et al., 1998). In the present study, recipient genotypes with contrasting levels of C18:3 produced the same level of the target fatty acids. However, the expression of the TE in recipient genotypes with different C22:1 levels resulted in significantly different levels of the target fatty acids. Enhanced levels of the target fatty acids in low C22:1 recipient genotypes, compared to in high C22:1 recipient genotypes, were detected in transgenic F_1 seeds and DH lines with different C22:1 levels originating from different crosses, as well as in DH lines with contrasting C22:1 levels developed from the same cross.

The increase in the level of the target fatty acids due to expression of the TE transgenes in low C22:1 recipient genotypes compared to the expression in high C22:1genotypes was mainly caused by the reduction of C22:1 in the total fatty acids. The level of a fatty acid is reported as the percentage of the total fatty acids in *Brassica* (Hougen and Bodo, 1973; Pleines and Friedt, 1989; Somerville, 1993). Thus, as the percentage of one fatty acid decreases, the percentages of the other fatty acids would be anticipated to rise if the regulation of the fatty acid synthesis was not changed. Although

the low C22:1 DH lines showed higher levels of the target fatty acids than the high C22:1 DH lines, the difference in the target fatty acid level between the low and high C22:1 lines was no more than expected due to the reduced percentage of C22:1 in the total fatty acids in the low C22:1 genotypes. It indicates that the reduction in the C22:1 level in the low C22:1 lines did not result in an increased availability of the acyl-ACP substrates for the target fatty acids of the TE in relation to the substrates for the other fatty acids in the seed oil.

In addition to the C22:1 level, differences between the recipient genotypes in other genetic factors have been associated with the variation in the target fatty acid level. As observed in some studies of the fatty acid composition of non-transgenic oilseed crop species (Kinoshita et al., 1998; Narvel et al., 2000; Rahman et al., 1997;Thomas and Kondra, 1973), the target fatty acids of the TE were mainly, but not completely, controlled by the genotype of the embryo in the seeds. The influence of maternal plant on the target fatty acids was detected by comparing F_1 seeds of reciprocal crosses between transgenic lines and two non-transgenic cultivars, AC Excel and Mercury. The difference between reciprocal F_1 seeds might can be due to the different sporophyte genotypes of maternal plants (maternal effect) and /or different cytoplasms (cytoplasmic effect) (Thomas and Kondra, 1973). Since the DH lines developed from reciprocal crosses were not significantly different, cytoplasmic effect on the target fatty acid level was excluded. Agronomic or physiological characters have been reported to influence the fatty acid composition of non-transgenic *B. napus* plants (Pleines and Friedt. 1989; Thomas and Kondra, 1973; Wilmer et al., 1996). Therefore, the maternal effect observed with F_1 seeds probably represented differences in such traits between the transgenic parental lines and the two cultivars.

Influence of homology-dependent gene silencing (HDGS), which is based on the interaction of homologous sequences in the genome (Matzke et al., 1999; Meyer et al., 1996), was not observed in this study with the TE transgenes. The promoter(1.7 kb) and terminator (1.2 kb) of the four TE transgenes tested in this study were the same (Jones et al., 1995; Voelker et al., 1996, 1997). Moreover, these TE genes share high homology in the DNA sequences (Jones et al., 1995), e.g. the bay- and cuphea-TE sequences average 61% identity in part (ca. 1 kb) of the coding regions (Jones

et al., 1995). However, transgenic seeds containing the bay-TE and any of the other three TE transgenes co-expressed the two TE. Thus, introduction of an additional TE with homology to the previously integrated TE does not necessarily result in co-suppression. Over-expression of the homologous, endogenous Δ12-desaturase transgene in *B. napus* has been reported (Hitz et al., 1995).

Co-expression of two TE transgenes could result in accumulation of the target fatty acids of the two TE in the seed oil at the same levels as found in the seeds expressing each TE alone. For example, in transgenic seeds carrying both the bay-TE and the cuphea-TE transgenes, the levels of the target fatty acids C12:0 and C16:0 of the two TE were not significantly different from the C12:0 level of the bay-TE hemizygous seeds and the C16:0 level of the cuphea-TE hemizygous seeds, respectively. Therefore, co-expression of two different TE transgenes provides breeders with an additional approach to develop *B.napus* cultivars with a modified fatty acid composition in the seed oil, e.g. oils with high levels of both C12:0 and C16:0.

6.2 Effects of Genomic Position and Copy Number of TE Transgenes on the Target Fatty Acids

Three loci of the cuphea-TE transgene and four loci of the elm-TE transgene were analyzed for the effect of genomic position on the level of the target fatty acid C16:0 in DH lines. No significant difference was detected with the three cuphea-TE loci. Similarly, no significant positional effect has been detected with other transgenes (Gendloff et al., 1990; Hobbs et al., 1990, 1993). However, DH lines with the elm-TE transgene at different genomic sites showed significantly different C16:0 levels. DH lines with only Locus E-1 or E-IV had a more than 2-fold difference in the C16:0 level (10.8% and 25.2%, respectively) although each of the loci had one copy of the elm-TE transgene. In addition, DH lines with only Locus E-III or E-IV showed an 1.3-fold difference in the C16:0 level (28.7% and 36.7%, respectively) although both the loci had two copies.

The effect of the genomic position of transgenes on the expression level has been widely observed among primary transformants from independent transformation events (Allen et al., 2000; De Neve et al., 1999; Gendloff et al., 1990). The chromatin structure (heterochromatin vs. euchromatin), existence of endogenous regulator sequence such as enhancer, and discrepancies in the GC content

between the transgene and the flanking sequences at the integration sites are believed to influence transgene xpression (Allen et al., 2000; Chandler and Vaucheret, 2001; Fagard and Vaucheret, 2000). Significant differences in the level of the fatty acids targeted by the elm-TE transgene between DH lines with the transgene at different loci suggested a positional effect on the expression, an indication of differences in the flanking sequences at the genomic sites. Similar levels of the target fatty acid C16:0 in the DH lines with the cuphea-TE transgene at different loci did not exclude the possibility of the influence from the flanking sequences; instead, it could indicate that the flanking sequences at those sites had a similar effect on the expression. Similarly, no significant positional effect was detected in some cases with other types of transgenes (Gendloff et al., 1990; Hobbs et al., 1990, 1993).

The effect of copy number of the TE transgenes on the level of the target fatty acids was demonstrated by comparisons between SP seeds and F_1 seeds, between transgenic DH lines with different numbers of the cuphea-TE transgene, as well as between lines with different numbers of the elm-TE transgene in this study. Dosage effect of transgenic copies has been detected by analysis of hemizygous and homozygous transgenic plants in studies with other transgenes (Azhakanandam et al., 2000; Beaujean et al., 1998; Tenllado and Diaz Ruiz, 1999).

The mean level of the target fatty acid C16:0 in cuphea-TE DH lines increased as the copy number increased from one to five copies per haploid genome. For example, DH lines with five copies had 26.1% C16:0 on average, which was significantly higher than the lines with only one copy (15.7% C16:0 on average). Based on 83 DH lines with from one to five copies of the cuphea-TE transgene, copy number and the level of the target fatty acid C16:0 had a positive linear correlation ($r = 0.77^{**}$), and copy number account for near 60% of the total variation in the level of the target fatty acid among the cuphea-TE DH lines. The positive linear correlation observed in this study was in agreement with previous reports regarding copy number and expression level with other transgenes (Gendloff et al., 1990; Hobbs et al., 1993; McCabe et al., 1999; van der Hoeven et al., 1994). The remainder of the total variation in the target fatty acid level which could not be explained by copy number of the TE transgene could be due to the variation such as observed between plants within

the same DH lines. Variation from plant to plant of the same TE transgenic DH lines was demonstrated by analysis of plants of eight transgenic DH lines. The variation between genetically identical plants has been ascribed to developmental and experimental errors (Bhattacharyya et al.,1994).

Study on the elm-TE transgenic DH lines showed that the achievement of an enhanced expression level of the target fatty acids by addition of extra copies depended on the loci involved, and also on the level already achieved. DH lines with four copies of the elm-TE transgene at two loci (Locus E-II and E-III) showed a mean C16:0 level of 35.2%, which was significantly higher than 28.7% C16:0 for the lines with two elm-TE transgene copies at Locus E-II, but not significantly different from 36.2% C16:0 for the lines with two elm-TE copies at Locus E-III. These results suggest that when the fatty acid level was relatively high, e.g. 36.2% C16:0 for the lines with two copies at Locus E-III, an addition of extra elm-TE transgene copies could not further increase the target fatty acid level. When the target fatty acid level was relatively low, e.g. 28.7% C16:0 for the lines with two copies at Locus E-II, the C16:0 level could be increased by increasing the number of elm-TE transgene copies and/or by enhancing expression of the transgene at different loci (e.g. Locus E-III). Thus, there appeared to be an upper limit of around 36.2% C16:0 observed in this study and 33% C16:0 in the primary transformants (Voelker et al., 1997) for elm-TE transgenic lines, above which the fatty acid level could not be increased by the addition of extra elm-TE transgene copies.

The existence of an upper limit for the target fatty acid level has been observed in a study with *B. napus* transgenic plants carrying the bay-TE transgene by analysis of the activity of the enzyme and the level of lauric acid (C12:0) targeted by the enzyme (Voelker et al., 1996). The explanation for the upper limit is that other enzymes in the fatty acid biosynthetic pathway and TAG assembly, e.g. β-ketoacyl ACP synthase (KAS) and aycltransferase (LPAAT), is inappropriate for further increase in the level of the target fatty acid in the *B. napus* genetic background (Eccleston and Ohlrogge, 1998; Hawkins and Kridl, 1998; Voelker et a l., 1997; Wiberg et al., 1997). The positive linear correlation observed between copy number of the cuphea-TE transgene and the level of the target fatty acid C16:0 could represent a similar relationship between the cuphea-TE enzyme activity and

the fatty acid level as observed with the bay-TE transgenic lines by Voelker et al. (1996). All the cuphea-TE transgenic lines produced less than 40% C16:0 in this study; thus, the activity of the cuphea-TE enzyme was still a limiting factor so that the increase in the activity by expression of more copies could result in higher levels of C16:0. However, for the elm-TE transgenic lines, the upper limit of the target fatty acid was lower than the upper limit for the bay-TE and the cuphea-TE transgenic lines. Expression of the elm-TE transgene at an appropriate genomic position with a limited number of copies (e.g. Locus E-III with two copies) could be enough for the target fatty acid to reach the upper limit level so that addition of more copies could not further increase the level of the target fatty acid C16:0.

6.3 Stability of TE Transgenic DH Lines

Several aspects in the stability of the expression of TE transgenes were investigated, including the influence of in vitro culture process on the stability, expression over generations and in different growing conditions.

No DH plants with a silenced TE transgene were detected although an in vitro culture process was involved in the development of the DH plants. It has been reported that, during in vitro culture, DNA methylation may occur, which could alter chromatin structures, thus leading to variation in transgene expression (Brown et al., 1990; Kaeppler and Phillips, 1993; Kaeppler et al., 2000; Olhoft, 1996). Gene silencing due to in vitro culture has been observed (Kaeppler et al., 2000). In the present study, more than 300 DH plants were developed from embryos that have undergone selection for the selectable marker gene with hybrid plants hemizygous for the bay- or the cuphea-TE transgenes being the microspore donors. Most of the DH plants (95%) expressed the TE transgene. The remaining plants did not show the expected transgenic phenotype since they did not carry the TE transgene, with some of the plants being regenerated from embryos escaped from the kanamycin selection and the others carrying incomplete T-DNA copies that did not have the TE transgene. No plants with a silenced TE transgene were detected. Therefore, an in vitro culture period involved in the DH line development process does not necessarily induce silencing of a TE transgene. In addition, the culture process did not increase variation in the expression level. DH lines for which

the chromosome number was doubled by microspore treatment (MT) did not show enhanced variation from plant to plant compared to the lines by root treatment (RT), indicating that mutations which could influence the variation of the expression of the TE did not occur during the in vitro culture period (Section 5.4). These results confirmed the study by Voelker et al. (1996) excluding the influence of in vitro culture on the expression of the bay-TE transgene.

High stability in the level of the target fatty acids of TE transgenic DH lines was observed in concurrently grown plants of different generations. The levels of the fatty acids targeted by the four TE transgenes remained the same for most of the transgenic DH lines carrying one of the TE transgenes over consecutive generations tested, two generations (DH_3 and DH_4 seeds) for the bay-TE and the cuphea-TE transgenic lines and three generations (DH_3, DH_4 and DH_5 seeds) for the elm-TE and the nutmeg-TE transgenic lines. The stability was not affected by the in vitro culture process and the recipient genotype. There are numerous reports showing stable expression of various transgenes over generations (Fearing et al., 1997; McCabe et al., 1999; Scott et al., 1998), including the bay-TE transgenes in *B. napus* plants (Voleker et al.,1996). The expression level of a transgenic CryIA(b) gene, for example, in *Bt* maize lines, were stable over successive backcross generations, with no significant differences among BC_1, BC_2, BC_3 and BC_4 populations planted concurrently (Fearing et al., 1997). Unstable expression as caused by PTGS has been associated with the production of special RNA signals, e.g. antisense RNA or double-stranded RNA molecules. The production of the signals could be due to the existence of a promoter located downstream of, or within, a gene whenever transcription is driven in an opposite direction, or due to transcription of inverted repeats (Meins et al., 2000; Wassenegger, 2000). The stable expression of the TE transgenes could be an indication of no such promoters and inserts of inverted repeats existing in the transgenic lines.

The growing condition of TE transgenic plants could significantly influence the target fatty acids of the TE. The influence of growing condition on the expression of the elm-TE, nutmeg-TE and cuphea-TE transgenes was detected by comparing the GH and GR grown plants, as well by comparing plants exposed to different temperatures during seed development. The elm-TE and the

cuphea-TE transgenic lines showed a higher level of the target fatty acid C16:0 at a high temperature condition (25/20 °C, day/night) than at low temperatures (20/15 °C), not a reduced expression level as expected with gene silencing commonly reported to reduce the expression (Köhne et al., 1998; McCabe et al., 1999). The effect of temperature on the target fatty acid could be caused through a distinct mechanism in transgenic plants compared to non-transgenic plants. Studies with conventional *B. napus* cultivars have demonstrated that increased temperature led to enhanced levels of C18:1 (Deng and Scarth, 1998; Pritchard et al., 2000; Wilmer et al., 1996), whereas in this study the increase in the target fatty acids of the TE transgenes was accompanied by a decrease in the C18:1 level.

6.4 Recommendations for Further Study

Several issues are proposed for further studies for better understanding of the expression of the TE transgenes in the *B. napus* genetic background:

1) The relationship between the expression of the TE transgenes and agronomic performance. F_1 seeds from reciprocal crosses of the transgenic parents with AC Excel or Mercury showed maternal effect on oil quality, which could be due to different agronomic characteristics, such as days to flower, of the two parents. Further study is needed to determine the genetic relationship between growth characters and the expression of the TE.

2) The possibility of increasing the level of the target fatty acid C16:0 of the cuphea-TE by increasing the copy number in the cuphea-TE transgenic lines. The DH lines with five copies of the cuphea-TE transgene showed a significantly higher mean C16:0 level than the lines with less copies. Therefore, there is a possibility of obtaining increased C16:0 levels by introducing extra copies of the TE transgene into the transgenic DH lines. Increase of the copy number could be achieved by re-transformation of the transgenic DH lines or by crossing transgenic lines from independent transformation events.

3) The influence of KAS II activity on the level of the target fatty acids. Bay-TE transgenic lines showed an upper limit of around 60% C12:0 in the original transformation experiment (Voelker et al., 1996) and a maximum level of 48% C12:0 in this study. One of the limiting factors for further increase in the target fatty acid is the poor affinity of acyltransferase LPAAT to saturated fatty acids

in the *B. napus* genetic background (Frentzen, 1993; Harwood and Page, 1994; Töpfer et al., 1995). This limits the incorporation of saturated fatty acids into one of the three positions in the glycerol backbone, the *sn*-2 position, resulting in a theoretical upper limit of 66%. The elm-TE transgenic lines showed a much lower upper limit (ca. 36% C16:0) than the bay-TE lines, above which the target fatty acid level could not be increased by expression of extra copies of the TE transgene. Competition of KAS II, the enzyme catalyzing the elongation of C16:0-ACP to C18:0-ACP, with the TE for the substrate C16:0-ACP is likely to be another limiting factor. Therefore, sense or anti-sense repression of the endogenous KAS II gene could probably increase the level of the target fatty acid C16:0 of the elm-TE up to the theoretical limit level.

4) DNA polymorphism of the genes coding for the enzymes in plant fatty acid biosynthesis and the significance of the polymorphism in the accumulation of the target fatty acids. The DH plants with the same copy numbers of the cuphea-TE or the elm-TE transgene showed wide variation in the level of the target fatty acid. The variation could be caused by developmental or experimental error, or by differences in those enzymes that influence the accumulation of the target fatty acids among the plants. In sunflower, examination of the desaturase genes with eight inbred lines showed that the length and nucleotide sequence of introns, as well as the 5'-untranslated region are polymorphic (Hongtrakful et al., 1998). In safflower, two C18:0-ACP cDNA TE isoforms showed similar but not completely the same specificity, with one being less discriminating against C16:0- and C18:0-ACP than the other although both prefer C18:1-ACP (Knutzon et al., 1992). Thus, it is possible that the polymorphism of fatty acid genes contributes to the variation in the fatty acid composition.

5) Confirmation of the effect of growing environment on the target fatty acids using field trials. Comparative analyses of DH lines growing in controlled environment indicated that growing environment can significantly influence the target fatty acids. The next step is to conduct field trials to determine the effect of environment on commercial production of TE transgenic cultivars.

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