Regulation of erucic acid accumulation in oilseed rape (*Brassica napus* L.):

effects of temperature and abscisic acid.

Promotor: dr. L.H.W. van der Plas
Hoogleraar in de Plantenfysiologie

Co-promotor: dr. J.P.F.G Helsper
wetenschappelijk onderzoeker plantenfysiologie
AB-DLO Wageningen

Regulation of erucic acid accumulation in oilseed rape (*Brassica napus* L.): effects of temperature and abscisic acid.

Jeroen A. Wilmer

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van de Landbouwuniversiteit Wageningen,

dr. C.M. Karssen,

in het openbaar te verdedigen

op donderdag12 juni 1997

des middags te half twee in de Aula

ISN 937008

Omslag: Volwassen, ± 800 °C·day oude, microspore-afgeleide embryo's van *Brassica napus* L. cv Reston. (foto AB-DLO)

ISBN 90-5485-695-5

Druk: Grafisch Service Centrum van Gils B.V., Wageningen

Stellingen

1. Regulatie van de vetzuursamenstelling in de olie van koolzaad is een kwantitatief proces.
   Dit proefschrift

2. De elongase activiteit bepaalt de hoeveelheid erucazuur die wordt gevormd, niet het erucazuurgehalte in de olie van koolzaad.
   Dit proefschrift

3. De overeenkomst in het effect van temperatuur op het erucazuurgehalte van olie in zaden en MDE's van koolzaad is, gezien de onderliggende verschillen in reactie op temperatuurverandering van de twee systemen, niet meer dan toevallig.
   Dit proefschrift

4. Produktie van erucazuur is een neutrale mutatie.
   Dit proefschrift

5. Oleoyl-CoA is het directe substraat voor elongatie in koolzaad.
   Hlousek-Radjocic *et al.*, Plant J. 8: 803-809 (1995), dit proefschrift.

6. De efficiëntie van een literatuursearch met de computer is omgekeerd evenredig met het aantal homoniemen voor elke zoekterm en het aantal synoniemen voor elke uitsluiting.

7. De omvang van de "verloren generatie" onder academici van na invoering van de wet twee-fasen structuur reduceert ieder post-doc beleid tot niet meer dan een gebaar.

8. Een spellingshervorming waarbij volledig voorbij wordt gegaan aan de gesproken taal zal niet leiden tot een verbetering van de schrijfprestaties; de fouten zullen zich slechts verplaatsen.

9. Onderwijs aan kinderen van asielzoekers is een niet geregistreerde vorm van speciaal onderwijs, en als zodanig de grootste invulling van de "weer samen naar school" filosofie.

10. Het recent geconstateerde feit dat de publieke omroep geen aandacht besteedt aan het carnaval geeft dit feest een kans terug te keren waar het behoort: in de geest en de kroegen en straten van de zuidelijke gewesten.

11. Gezien hun gepostuleerde afmetingen en afkeer van open ruimtes, is een rol van kabouters bij de bestuiving van planten hoogst onwaarschijnlijk.

    R. Poortvliet: de Kabouter. Van Holkema & Warendorf, Bussum (1976); F.A. Hoekstra: Stellingen bij "Vitality and metabolic properties of binucleate and trinucleate pollen species upon dehiscence" (1979).

Stellingen behorende bij het proefschrift "Regulation of erucic acid levels in oilseed rape (*Brassica napus* L.): effects of temperature and abscisic acid" door Jeroen Wilmer.

Wageningen, juni 1997

Voorwoord

Geen enkel proefschrift komt tot stand zonder de hulp van anderen dan de promovendus. Ook in dit geval is dat zo, al is het met een klein verschil: dit keer geen studenten of analisten die proeven hebben uitgevoerd, maar wel een aantal collega's die gezorgd hebben voor begeleiding, werksfeer en goede ideeën bij het oplossen van de dagelijkse problemen.

Allereerst gaat daarbij mijn dank naar de mensen die gedurende de afgelopen jaren mijn begeleiders zijn geweest. Prof. van der Plas, Linus, jouw continue aanwezigheid en persoonlijke aandacht hebben er sterk toe bijgedragen dat het project is afgerond binnen een redelijke termijn. Dr. Jordi, Wilco, jouw naam is uiteindelijk niet boven een van de hoofdstukken of artikelen gekomen, maar ik waardeer het zeer hoe je vooral in het begin van deze periode je hebt ontfermd over een verweesde AiO. Jouw ideeën hebben geholpen bij het opnieuw opzetten van het project toen bleek dat er heel weinig overbleef van de oorspronkelijke opzet. Dr. Helsper, Hans, bedankt voor het overnemen van mijn dagelijkse begeleiding in de loop van het eerste jaar en je inspanningen gedurende de afgelopen vier jaar.

I would like to thank Dr. Mukherjee from Münster, who freely shared his ideas and advice in developing the project. I greatly enjoyed our discussions at the institute and over lunch afterwards on the occasions we were able to visit you. The chapters 4 and 5 of this thesis owe their existence to the hospitality of the people at the "Laboratoire de Biogenèse Membranaire" in Bordeaux, who gave me the opportunity to do crucial elongase assays with their group. Thank you Prof. Cassagne, René Lessire and Fred Domergue.

Daarnaast zijn er een aantal mensen die bijgedragen hebben in praktische, maar niet onbelangrijke zaken zoals Adrie Kooijman, die de koolzaadplanten heeft verzorgd, Evert Davelaar en Jacques Davies, die hebben geholpen bij het opzetten van analyses. En dan zijn er de collega's van de afdeling

Biochemie en Celfysiologie, later Plantenfysiologie, met wie ik menige discussie heb gehad over onderzoek en alles wat daar ook maar aan kon raken. Allen, en met name Iris en Anja, dank voor de goede sfeer.

Tot slot, maar zeker niet in het minste, zou dit proefschrift niet tot stand gekomen zijn zonder het geduld van degene die de afgelopen jaren met mij heeft moeten leven. Sigrid, zonder jouw steun en goede zorgen was dit niet gelukt.

Ik draag dit proefschrift op aan mijn opa, Jan Wilmer, die er niet meer bij mag zijn, maar die apetrots zou zijn dat zijn kleinzoon verder gegaan is in Wageningen, waar hij een aantal jaren gewerkt heeft.

Contents

List of abbreviations

12:0	lauric acid
16:0	palmitic acid
18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid
20:1	eicosenoic acid
22:1	erucic acid
ABA	abscisic acid
ACP	acyl carrier protein
CoA	Coenzyme A
DAG	diacylglycerol
DAGAT	diacylglycerol acyl-CoA acyltransferase
FAME(s)	fatty acid-methylester(s)
G-3-P	glycerol-3-phosphate
G-3-PAT	glycerol-3-phosphate acyl-CoA acyltransferase
GC	gas chromatography
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyl-CoA acyltransferase
MDE(s)	microspore-derived embryo(s)
PA	phosphatidic acid
PC	phosphatidyl choline
TAG	triacylglycerol
TLC	thin-layer chromatography
TS	temperature sum
VLCFA(s)	very long chain fatty acid(s)

Chapter 1

INTRODUCTION

1.1 Vegetable oils: a commodity

World-wide about 72 million tonnes of vegetable oils and fats were produced in the 1995/1996 growing season (USDA, 1996). The major crops, responsible for nearly 80% of the total production, are soybean, oil palm, oilseed rape and sunflower. Other species used for oil production are olive, coconut and peanut. The majority of these oils and fats, consisting of triacylglycerols, is used in food applications, which implies that the fatty acid composition of these oils has to fit both human diet recommendations and the actual food product it is used in. Predominant fatty acids in edible oils are oleic (18:1), linoleic (18:2), linolenic (18:3) and palmitic (16:0) acid, with smaller amounts of stearic (18:0) and sometimes lauric (12:0) acid. The exact oil composition desired depends upon the product it is used in: short chains and saturated fatty acids give rise to solid fats, but are less desirable from a dietary point of view. Polyunsaturated fatty acids like linolenic, which make fluid oils, are preferable or even essential in the human diet, but are more prone to oxidative degradation.

Apart from these straight chain, unsubstituted, medium and long chain fatty acids a large number of other fatty acid types can be found in plant oils. Chain lengths can range from 8 to 24 carbons (table 1.1) and may contain hydroxyl and epoxy groups or specific types of desaturations. Also wax esters of long chain fatty acids and fatty alcohols, another form of oil, can be found, like in jojoba. In this large and varied group of specific oils various applications are possible: short chain fatty acids (8 to 12 carbons) are used as detergents, jojoba wax esters in cosmetics and very long chain fatty acids in high quality lubricants and diesel fuel. However, production of these oils is not always economically feasible due to the characteristics of the plant species producing them, resulting for instance in low yields, poor crop performance or specific requirements for extensive processing.

Table 1.1: Structure and common names of important fatty acids

Name:	Abbreviation:	Structure:
Capric acid	10:0	$CH_3{\cdot}(CH_2)_8{\cdot}COOH$
Lauric acid	12:0	$CH_3{\cdot}(CH_2)_{10}{\cdot}COOH$
Myristic acid	14:0	$CH_3{\cdot}(CH_2)_{12}{\cdot}COOH$
Palmitic acid	16:0	$CH_3{\cdot}(CH_2)_{14}{\cdot}COOH$
Stearic acid	18:0	$CH_3{\cdot}(CH_2)_{16}{\cdot}COOH$
Oleic acid	18:1 (Δ9)	$CH_3{\cdot}(CH_2)_7{\cdot}CH{:}CH{\cdot}(CH_2)_7{\cdot}COOH$
Linoleic acid	18:2 (Δ9, 12)	$CH_3{\cdot}(CH_2)_3{\cdot}(CH_2{\cdot}CH{:}CH)_2{\cdot}(CH_2)_7{\cdot}COOH$
α-Linolenic acid	18:3 (Δ9, 12, 15)	$CH_3{\cdot}(CH_2{\cdot}CH{:}CH)_3{\cdot}(CH_2)_7{\cdot}COOH$
γ-Linolenic acid	18:3 (Δ6, 9, 12)	$CH_3{\cdot}(CH_2)_3{\cdot}(CH_2{\cdot}CH{:}CH)_3{\cdot}(CH_2)_4{\cdot}COOH$
Arachidic acid	20:0	$CH_3{\cdot}(CH_2)_{18}{\cdot}COOH$
Gondoic acid	20:1 (Δ11)	$CH_3{\cdot}(CH_2)_7{\cdot}CH{:}CH{\cdot}(CH_2)_9{\cdot}COOH$
Erucic acid	22:1 (Δ13)	$CH_3{\cdot}(CH_2)_7{\cdot}CH{:}CH{\cdot}(CH_2)_{11}{\cdot}COOH$
Nervonic acid	24:1 (Δ15)	$CH_3{\cdot}(CH_2)_7{\cdot}CH{:}CH{\cdot}(CH_2)_{13}{\cdot}COOH$

In recent years the capacity to produce some of these specific oils has been introduced in established crops by means of genetic modification. So far experiments with oilseed rape have been successful in producing oil with increased levels of stearic acid (Knutzon *et al.*, 1992a) or oils containing lauric acid (Voelker *et al.*, 1996). The plants resulting from the latter experiments were first planted on a large scale in the USA as a commercial crop in the '94-'95 growing season.

Erucic acid (22:1) is a very long chain fatty acid which naturally occurred in oilseed rape and has recently gained considerable interest because of its non-food applications. Because of its presumed role in increasing the risk of coronary disease in humans (see chapter 6, general discussion), classical breeding techniques had been applied to obtain modern erucic acid free canola-type cultivars. The physical properties related to its chain length of 22 carbons make erucic acid a valuable component of waxes and polishes, hydraulic fluids and lubricants. The position of a single double bond in the C22-chain allows oxidative cleavage resulting in a 13-carbon bifunctional acid. This moiety can be used in the production of nylon 1313, a high quality, technical

plastic. Recently efforts have been made to increase the level of erucic acid in modern cultivars of oilseed rape both by classical breeding (*e.g.* Scarth *et al.*, 1995) and molecular genetic techniques (Lassner *et al.*, 1995).

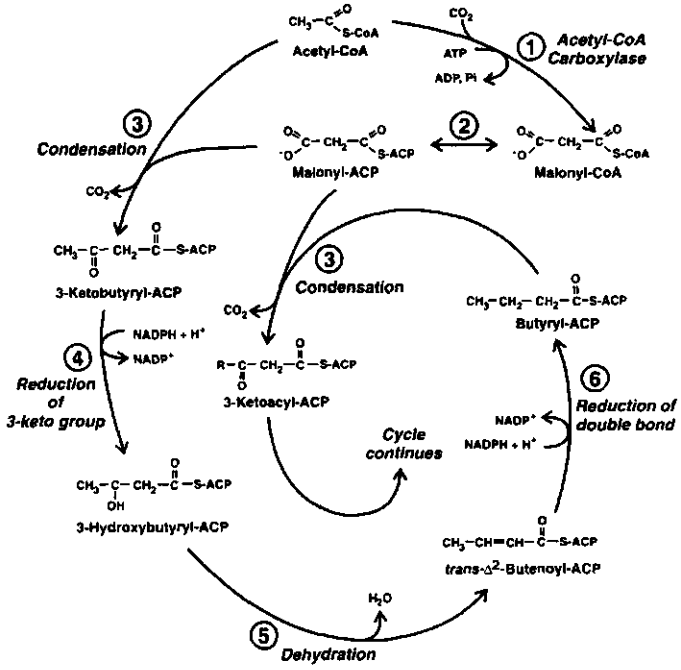


Fig 1.1: Schematic representation of the reactions involved in saturated fatty acid synthesis. (Reprinted from Ohlrogge & Browse, 1995).
Acetyl-CoA is the basic building block of the fatty acid chain and enters the pathway both as a substrate for acetyl-CoA carboxylase (1) and as a primer for the initial condensation reaction (3). Reaction 2, catalysed by malonyl-CoA:ACP transacylase, transfers malonyl from CoA to form malonyl-ACP, which is the carbon donor for all subsequent elongation reactions. After each condensation, the 3-ketoacyl-ACP product is reduced (4), dehydrated (5), and reduced again (6), by 3-ketocayl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, respectively.

1.2 Enzymes involved in oil synthesis

Oil synthesis requires the coordinated activity of a large number of enzymes. Fatty acids are synthesised *de novo* by a dissociable enzyme complex (prokaryotic, type II) by sequential condensation of C2-units derived from malonyl-Coenzyme A. Next desaturation and other modifications may take

place, at the level of CoA-esters, or after incorporation in phosphatidylcholine. Finally fatty acids have to be incorporated into "functional" lipids, like galactolipids, phospholipids or triacylglycerol (oil). In recent years a large number of the enzymes and genes involved in this biosynthetic pathway have been isolated either from green tissues or from developing seeds (table 1.2). In this thesis the focus will be on the fatty acid modifying and incorporating enzymes in the endoplasmic reticulum and other extra-plastidial membranes leading to the production of triacylglycerol (oil), and especially the regulation of these pathways. A separate set of enzymes with similar activities is present in the plastids (for a recent review, see Ohlrogge & Browse, 1995).

Fatty acid biosynthesis

The first step in oil production is *de novo* synthesis of fatty acids, which occurs in plastids of all plant tissues. Fatty acid synthesis starts with the condensation of acetyl-CoA and malonyl-acyl carrier protein (malonyl-ACP, fig 1.1) by ß-ketoacyl-synthetase III. In each round of elongation from this primer, incorporation of a 2-carbon unit from malonyl-ACP also requires two reduction steps and a dehydration step. It appeared that three different condensing enzymes are used in producing 18-carbon fatty acids (Schuch *et al.*, 1994, Jones *et al.*, 1995) with ß-ketoacyl-synthetase I performing condensations leading from C4 to C16 and ß-ketoacyl-synthetase II performing the last condensation resulting in an 18-carbon chain. In other studies it has been shown that component enzymes can complement mutants in *E. coli* and *vice versa* showing that the various enzymes are conserved both in function and in general mode of regulation over prokaryotic organisms (Kater *et al.* 1994, Verwoert *et al.* 1995a/b), as plastids are thought to be according to the endosymbiont theory. However, little is known about either the stoichiometry of the fatty acid synthase complex or the way in which activity of the different enzymes is coordinated.

Table 1.2: Enzymes involved in oil synthesis and plant species from which either the enzyme or the gene has been isolated.

Enzyme:	Isolated from:	Sequence[1]:	Reference:
Fatty acid synthetase:			
β-ketoacyl-synthetase I	*Hordeum vulgare*	Y	Kauppinen (1992)
β-ketoacyl-synthetase II	*Ricinus communis*	Y	Genez (1993)
β-ketoacyl-synthetase III	*Spinacia oleracea*	Y	Tai & Jaworski (1993)
(EC 2.3.1.41)	*Arabidopsis thaliana*	Y	Tai *et al.* (1994)
	Cuphea wrightii	Y	Slabaugh *et al.* (1995)
β-ketoacyl-reductase	*Brassica napus*	Y	Martinez-Rivas *et al.* (1993)
β-hydroxyacyl-dehydratase	--	N	
trans-enoyl-reductase	*Brassica napus*	Y	Cottingham *et al.* (1988)
	Brassica napus	Y	Fawcett *et al.* (1994)
Δ9 stearoyl-ACP desaturase	*Spinacia oleracea*	Y	Nishida *et al.* (1992)
(EC 1.14.99.6)	*Ricinus communis*	Y	Fox *et al.* (1993)
	Brasica napus	Y	Slocombe *et al.* (1992)
	Thunbergia alata	Y	Cahoon *et al.* (1994)
	Sesamum Indicum	Y	Yukawa *et al.* (1996)
thioesterase	*Carthamus tinctorius*	Y	Knutzon *et al.* (1992b)
(EC 3.1.2.14)	*Cuphea sp.*	N	Dormann *et al.*(1993)
	Cucurbita moschata	N	Imai *et al.* (1992)
ACP	various species, see:		Töpfer & Martini (1994)
Fatty acid modifying enzymes:			
Desaturases:			
Δ4 (16:0-ACP dependent)	*Coriandrum sativum*	Y	Cahoon *et al.*(1994)
Δ12 (ω6)	*Arabidopsis thaliana*	Y	Okuley *et al.* (1994)
	Glycine max	Y	Heppard *et al.* (1996)
Δ15 (ω3)	*Arabidopsis thaliana*	Y	Arondel *et al.* (1992)
Δ6 (Δ12 dependent)	*Borago officinalis*	N	Griffiths *et al.* (1988)
Elongase:			
KAS	*Arabidopsis thaliana*	Y	James *et al.* (1995)
	Simmondsia chinensis	Y	Lassner *et al.* (1996)
Fatty acid incorporation, triacylglycerol assembly:			
G-3-P acyltransferase	--	N	
(EC 2.3.1.15)			
LPA acyltransferase	*Cocos nucifera*	N	Davies *et al.* (1995)
(EC 2.3.1.51)	*Limnanthes douglasii*	Y	Brown *et al.* (1995)
	Limnanthes alba	Y	Lassner *et al.* (1995)
PA phosphatase	*Brassica napus*	N	Kocsis *et al.* (1996)
(EC 3.1.3.4)			
DAG acyltransferase	*Brassica napus*	N	Little *et al.* (1994)
(EC 2.3.1.20)			

[1]: Y: sequence available at the protein or (c)DNA-level, N: no sequence available.

Regulation of fatty acid synthesis itself in leaves seems to occur at the level of acetyl-CoA carboxylase. This enzyme catalyses the formation of malonyl-CoA, the precursor of malonyl-ACP. It is regulated by light (Post-Beittenmiller *et al.*, 1991) and by lipids, following a feedback mechanism (Shintani & Ohlrogge, 1995). Regulation of fatty acid biosynthesis in developing seeds is less well understood.

Generally chain elongation is terminated at 16 (palmitic acid and derivatives) or 18 carbon atoms. Most of the 18:0 synthesised this way is desaturated to 18:1 by a Δ9 stearoyl-ACP desaturase before being cleaved off from the ACP by a thioesterase and exported from the plastid. Thus, most plastids predominantly export 18:1, 16:0 and some 18:0, which are used for further modification and incorporation in glycerolipids. In some species like *Cuphea* sp. (Dormann *et al.*, 1993, Schuch *et al.*, 1993, Heise & Fuhrmann, 1994) a thioesterase prematurely cleaves the growing acyl chain from the ACP, resulting in production of short or medium chain fatty acids.

Fatty acid modification

In general, modification involves the formation of one or two more double bonds in the carbon chain by a Δ12- and a Δ15-desaturase to obtain the polyunsaturated linoleic (18:2) and linolenic (18:3) acids. This modification occurs in membranes on oleic acid incorporated in phosphatidylcholine (PC). An acyl exchange mechanism makes the polyunsaturated fatty acids available for other lipids. Most recent results indicate that desaturation of fatty acids does not occur in the ER as was previously thought, but in the plasma membrane (Murata, pers. comm.). The biochemical mechanism of desaturation has recently been substantially further elucidated, since models of the enzymes became available (Fox *et al.*, 1993, Shanklin *et al.*, 1994), but the physiological regulation of these enzyme activities is still unclear.

Two hypotheses have been postulated for the regulation of the desaturation level in membranes. The first hypothesis proposes that the level of desaturation is regulated directly by the properties of the membrane surrounding the enzyme. An increase in temperature would thus lead to increased membrane fluidity and decreased oxygen solubility, thereby reducing desaturase activity. A decrease in temperature would in the same way activate the enzyme (Trémolieres *et al.*, 1982). The second hypothesis states that fatty acid desaturation is regulated by flux control, where the relative amount of desaturase compared to total fatty acid production varies with conditions, thus resulting in different levels of desaturation (Browse and Slack, 1983). Both models contain a homeostatic component, in assuming that the physical properties of the membrane are preserved with changes in external conditions. Mutations in desaturase genes have been shown to lead to disturbance of membrane function when temperature changes (Hugley *et al.*, 1989, Kodama *et al.*, 1994, Miquel & Browse, 1994). Whether this type of regulation is functional in oil synthesis is not clear.

For other modifications like the formation of ricinoleic acid (12-hydroxyoleic acid) and the formation of other oxygenated derivatives the reaction mechanism has not yet been fully elucidated. Even less is known about the regulation of the enzymes performing these modifications, but most models propose a role for PC- bound fatty acids as in desaturation.

Another type of modification is the elongation of fatty acids beyond 18 carbons. In this type of modification, oleic acid is extended using malonyl-CoA in a sequence of reactions similar to *de novo* fatty acid biosynthesis. Like fatty acid synthesis this process requires a set of cooperative enzymes, in this case embedded in the ER membrane. So far only one gene involved in this process has been cloned, FAE1, encoding the condensing enzyme (KAS) of *Arabidopsis* (James *et al.*, 1995), and its homologue from jojoba (Lassner *et al.*, 1996). Depending on the plant species the products of the elongation can be

C20 and C22 saturated fatty acids, used in wax production, as shown for leek (Schneider *et al.*, 1993, Evenson *et al.* 1995) or C20 to C24 monounsaturated fatty acids which are observed in the oil of cruciferous plants (Taylor *et al.*, 1992, Creach *et al.* 1993). For the elongase system an increase in activity coinciding with the start of oil production has been shown (Taylor *et al.*, 1992), but little is known about the regulation of this increase in activity. A stimulatory effect of abscisic acid (ABA) on both elongase activity and oil production has been found in microspore-derived embryos (MDEs) of oilseed rape (Holbrook *et al.*, 1992), but these data have not been linked with levels of ABA *in vivo* or with a detailed study on the kinetics of the enzyme. Also some indication of a regulation by temperature has been inferred in developing seeds (Canvin, 1965) and MDEs of oilseed rape (Albrecht *et al.*, 1994). Canvin has shown that in mature seeds the level of 22:1 reached a maximum at 15 °C, with lower levels at both higher and lower temperature. Albrecht *et al.* mainly showed a shift in timing of oil and 22:1 accumulation in MDEs with changes in temperature.

Incorporation of fatty acids into glycerolipids

The pathways for the biosyntheses of extra-plastidial membrane lipids and of storage triacylglycerols (TAG) share a number of enzymes. In both pathways fatty acids are first esterified to glycerol-3-phosphate to form lysophosphatidic acid (LPA, fig 1.2), then a second fatty acid is added to form phosphatidic acid (PA) and subsequently phosphate is removed to form diacylglycerol (DAG). Most membrane lipids are derived from DAG, although some can be derived from PA. The only reaction specific for the formation of TAG is the addition of a third fatty acid to DAG. A shared pathway means that, like in fatty acid synthesis, a constitutive level of enzyme activities has to be present in all cells of a plant. When storage products are being formed the whole pathway must be

upregulated as the flux through the pathway must be increased enormously (95% of lipids in seeds can be oil).

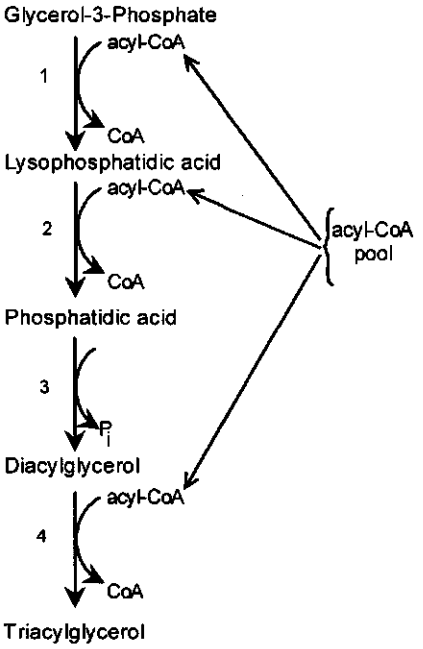


Fig 1.2: Schematic representation of the reactions involved in triacylglycerol bioassembly.
1: Glycerol-3-phosphate acyl-CoA acyltransferase (GPAT), 2: lysophospatidate acyl-CoA acyltransferase (LPAAT), 3: phosphatidate phosphatase, 4: diacylglycerol acyl-CoA acyltransferase (DAGAT).

The enzymes involved in this pathway have been characterised in a number of species (table 1.2) and show similar activities. The first acyltransferase (glycerol-3-phosphate acyl-CoA acyltransferase, GPAT) and the last (diacylglycerol acyl-CoA acyltransferase, DAGAT) seem to incorporate fatty acids mainly in the proportions in which they are available in the substrate pool, although some species preferentially incorporate saturated fatty acids at the first position of glycerol-3-phosphate (sn-1). The second acyltransferase (lysophosphatidate acyl-CoA acyltransferase, LPAAT), incorporating fatty acids at the sn-2 position, shows a much higher specificity and only incorporates

unsaturated fatty acids in oilseed rape (Bernerth & Frentzen, 1990), or generates two types of PA containing either two "seed specific fatty acids", like lauric acid in oil palm and *Cuphea* sp., or two long chain fatty acids normally found in membranes (Oo & Huang, 1989, Bafor *et al.*, 1990). These differences in substrate specificity of the enzymes incorporating fatty acids result in at least part of the typical fatty acid distributions found in triacylglycerols in oilseeds. Currently studies are in progress to explore the effects of transformation with genes encoding acyltransferases with altered specificity, on fatty acid composition in oils of various plant species, among which oilseed rape (Lassner *et al.*, 1995).

1.3 Scope of the thesis

To increase the value of rapeseed oil as an industrial feedstock, the fatty acid composition has to be of a constant and high quality. In this thesis we explore regulation of erucic acid accumulation in oil from oilseed rape by temperature and ABA. MDEs are used as a model system because of their small size compared with entire plants and the possibilities to manipulate growth conditions by adding compounds to the culture medium. In chapter 2 a comparison is made between seeds and MDEs regarding the effects of temperature on fatty acid composition. A method is described to enable comparison of the physiological age of material from the two systems. In chapter 3 the effects of ABA and its interaction with temperature is investigated. Chapter 5 reports an investigation of the role of methyl groups on the ring and of enantiomeric forms of the ABA molecule in physiological activity. The effects of temperature and ABA on acyltransferase activity and on the activity and properties of the elongase complex are described in chapter 4, and the activities are coupled to the levels of erucic acid and oil found in MDEs. In the last chapter the experimental data of this thesis are used to build a descriptive model for regulation of erucic acid levels in oilseed rape and the implications of

this model for manipulation of lipid metabolism are discussed. In this chapter we will also address the biological role of erucic acid in the seed and effects on plant fitness of changing the level of this fatty acid by changing temperature and ABA levels.

Chapter 2

EFFECT OF GROWTH TEMPERATURE ON ERUCIC ACID LEVELS IN SEEDS AND MICROSPORE-DERIVED EMBRYOS OF OILSEED RAPE, *Brassica napus* L.

Jeroen A. Wilmer, Johannes P.F.G. Helsper and Linus H.W. van der Plas

This chapter was published in: J. Plant Physiol. 147: 486-492 (1996)

Keywords

Brassica napus L.; erucic acid; fatty acid; growth temperature; microspore-derived embryos; seed development.

2.1 Summary

Effect of growth temperature on the fatty acid composition of tryglycerides was compared for developing seeds and microspore-derived embryos of rapeseed. Plants were grown under standard conditions and subsequently seed development was allowed to take place at 15 or 25 °C. The composition of seed oil of the low erucic acid cultivar Aurora and a high erucic acid Gulle-derived line were not affected by growth temperature. The cultivar Reston showed a 30% reduction in erucic acid level in seeds from plants grown at 25 °C as compared to seeds from plants grown at 15 °C. Seeds from plants, that were transferred once during development from 15° to 25° or *vice versa* after two thirds of their development had been completed, showed an oil composition similar to seeds that developed completely at the initial temperature. Seeds transferred before or during the period of maximum lipid synthesis showed oil compositions that were similar to oil formed at the final temperature or intermediate between those of seeds completely grown at 15 °C or 25 °C, respectively.

In microspore-derived embryos (MDEs) similar responses to temperature in the level of erucic acid could be observed. However, absolute levels of erucic acid in MDEs of both Reston and Gulle were 30-50% lower than in seed oil. Results are discussed in relation to possible regulatory mechanisms of temperature-induced changes in oil composition.

2.2 Introduction

The utilisation of vegetable oils for various applications increases continuously. Demands vary from cooking oils of high nutritional value to specific industrial

feed stocks and from short chain fatty acids to substituted and very long chain fatty acids. This led to studies on a number of potentially interesting plant species like *Crambe abyssinica* (Vollmann & Ruckenbauer, 1993), *Tropaeolum majus* (Pollard & Stumpf, 1980a), *Limnanthes alba* (Pollard & Stumpf, 1980b) and *Cuphea* spec. (Fuhrmann & Heise, 1993; Heise & Fuhrmann, 1994; Schuch *et al.*, 1993). These species all contain high levels of commercially valuable fatty acids but few of them are established crops. Studies were performed to see if established oilseeds, *e.g.* rapeseed, can properly incorporate the desired fatty acids in their seed oil (Battey & Ohlrogge, 1989). Theoretically rapeseed (*Brassica napus* L.) can contain up to 66% of erucic acid (22:1 Δ13) in seed oil, which is incorporated at positions sn-1 and sn-3 of the triglycerides (Bernerth & Frentzen, 1990). Cultivars approaching 60% of 22:1 are now in the nurseries of breeders.

Growth conditions hardly influence 22:1 levels (Schuster & Taghizadeh, 1980) with the exception of one factor, *i.e.* growth temperature. In a number of plant species higher temperatures lead to a reduction in polyunsaturated C18 fatty acids in the oil. This effect was correlated with changes in membrane fluidity and the availability of oxygen (Harris & James, 1969, Tremolières *et al.*, 1982). In triglycerides of rapeseed temperature mainly influences the levels of oleic (18:1) and erucic acids. Canvin (1965) reported maximum levels of 22:1 in oil of mature seeds from plants grown at 15 °C with reduced levels of 22:1 at both lower and higher temperatures, while a decrease in total oil content was observed from about 52 to 32% of seed dry weight with increasing temperature. In previous studies microspore-derived embryos (MDEs) have been reported to behave similarly to seeds in their development and oil accumulation (for a review see Taylor & Weber, 1994). Recent studies have reported some effects of culture temperature on developing MDEs. The rate of development at 15 °C was lower than at 25 °C, but the final oil composition was very similar for both conditions (Albrecht *et al.*, 1994, Möllers *et al.*, 1994).

In this study we have compared the effect of growth temperature on development and fatty acid composition of oil produced in both seeds and MDEs. MDEs were studied because they are a more effective experimental tool for physiological and biochemical studies, e.g. for the determination of enzyme activities involved in lipid biosynthesis (Taylor *et al.*, 1990). Since the developmental rate of MDEs and seeds is temperature-dependent we used temperature sum as a physiological time scale throughout. To determine developmental stages in which temperature may affect oil composition, seeds were collected from plant material that was exposed to a temperature change at different timepoints during development. Three genotypes differing in 22:1 level in seed oil were used to separate the effects on desaturation and elongation and to detect genotypic differences in responsiveness to changes in growth temperature.

2.3 Materials and Methods

Plant material.

Three genotypes of spring rape were used in our experiments: a low erucic acid type of German origin, Aurora, and two high erucic acid types, Reston, from the University of Manitoba, Canada, and a breeding selection from Gulle, obtained from Svalöf AB, Sweden (further referred to as "Gulle"). Plants were grown in the greenhouse in potting soil in 2-litre pots, at 20/17 °C (day/night) at a photo period extended to 16 hours with SON-T AGRO (Philips) to ensure induction of flowering. At the onset of flowering plants were transferred to growth chambers (16h photo period, 370 µmol $m^{-2}s^{-1}$ fluorescent light, 15 or 25 °C continuously) to allow seed development. Racemes were harvested for the induction of microspore-derived embryos also.

Developmental series and temperature switch.

Development of seeds and embryo material was described on a basis of temperature sum (TS in °C·day = number of days x mean temperature in °C, base temperature 0 °C). Flowers were tagged at anthesis and seeds were harvested from plants grown at 15 or 25 °C at intervals of about 100 °C·day. Some seeds were partially dissected to determine their developmental stage and the other seeds stored at -80 °C for analysis of the fatty acid composition of the oil (triglycerides).

Other plants were transferred during development from 15 to 25 °C or *vice versa* with 100 °C·day intervals. Seeds were harvested at maturity (about 950 °C·day).

Culture of microspore-derived embryos.

MDE culture was performed using a modification of the protocol used by Custers *et al.* (1994). Racemes were collected at the onset of flowering and buds of 3.4 to 3.6 mm length were selected. The buds were surface-sterilised in diluted commercial bleach (2% NaClO) and homogenised in a modified Lichter medium (NLN82, 13% sucrose without hormones, Gland *et al.*, 1988). After filtration over a 45 µm nylon mesh microspores were collected by centrifugation at 100 xg for 3 min, washed 3 times in the same medium, diluted to $4x10^4$ spores ml^{-1} and 1 ml portions of suspension were plated in 35 mm petri dishes. To induce embryogenesis the microspore suspension was exposed to 32 °C for three days and then to 25°C for another 4 days (200 °C·day). At this point embryos for 15 °C-treatments were transferred to that temperature. Embryos were cultured for another 100-900 °C·day until the desired temperature sum was reached. Embryos were rinsed with tap water and stored at -80 °C for analysis of the oil composition. Calculation of temperature sum started at the beginning of the 32 °C-treatment, because changes in protein patterns have been shown already after the first 8 hours of culture (Cordewener *et al.*, 1994).

Lipid extraction.

Seeds or embryos (50-700 mg) were homogenised using an Omni 2000 homogeniser with a 1 cm probe in 5 ml of methanol : chloroform : 0.01 N hydrochloric acid (2:1:0.8 by vol.), containing trimargarin as an internal standard. After shaking for 15 min the extract was transferred to a screw-capped centrifuge tube and chloroform and 0.01 N hydrochloric acid were added to obtain a mixture with a ratio 2:2:1.8 by vol. After thorough mixing the suspension was centrifuged to achieve phase separation. The aqueous phase was washed once with 1.5 ml synthetic organic phase, organic fractions were combined and stored under nitrogen at 4 °C until further analysis.

Thin-layer chromatography and transesterification of lipids.

Aliquots of chloroform-extracts were applied to 20 x 20 cm TLC plates which were developed in hexane : diethyl ether : acetic acid (70:30:1 by vol.). The plates were dried and lipids visualised with iodine vapour. Areas containing triglycerides were scraped off and the powder transferred to 3 ml 5% sulphuric acid in methanol. After flushing with nitrogen tubes were capped and incubated at 70 °C for three hours, with shaking every 15 min during the first 1.5 hours. Subsequently 3 ml of water and 3 ml of hexane were added and mixed by rotation for 15 min to extract the fatty acid methyl esters (FAMEs) into the organic layer. After centrifugation the organic layer was collected and the methanol/water mixture was washed with 1.5 ml hexane. The two organic fractions were combined and stored at 4°C for GC-analysis.

GC-analyses.

FAMEs were analysed using a capillary Chrompack CP9000 gas-chromatograph equipped with a 25 m x 0.32 mm CPwax 52 CB column and flame ionisation detection. Injector temperature was 260 °C, detector

temperature 300 °C, oven temperature was programmed from 190 °C (4.1 min) to 230 °C (4.4 min) at 20 °C min^{-1}. Helium was used as carrier gas at 85 kPa. FAMEs were identified on the basis of retention time and quantified by electronic integration with reference to methyl margarate, derived from the internal standard trimargarin.

Chemicals.

All solvents used were HPLC or reagent grade, trimargarin and FAME-standards were purchased from Sigma and TLC-plates (0.5 mm Silica gel 60 plates) from Merck.

Statistical treatment.

Data were fitted with sigmoidal curves reflecting the transitional nature of the processes studied. Four parameter-analysis of pairs of sigmoids was not possible due to a limited number of timepoints and small slopes of some curves. Therefore values from the start and end points of the sigmoids were compared using Student's t-test ($\alpha = 0.05$).

2.4 Results

Temperature sum as a measure of physiological age.

Table 1: Morphological development of seeds based on temperature sum (TS, mean day temperature x number of days).

TS (°C·day)	Embryo
200	very small, globular
300	torpedo
400	early cotyledonary
500	start of oil deposition
700	oil deposition nearly completed
800	start maturation
900	yellowing, seed coat brown
1000	mature: yellow and dry

When seeds grown at different temperatures were compared based on the number of days after anthesis, discrepancies were observed because seeds matured in about 40 days at 25 °C, but required more than 60 days for complete maturation at 15 °C (fig 1A). This discrepancy was circumvented when temperature sum was used as a measure of physiological age (fig 1B).

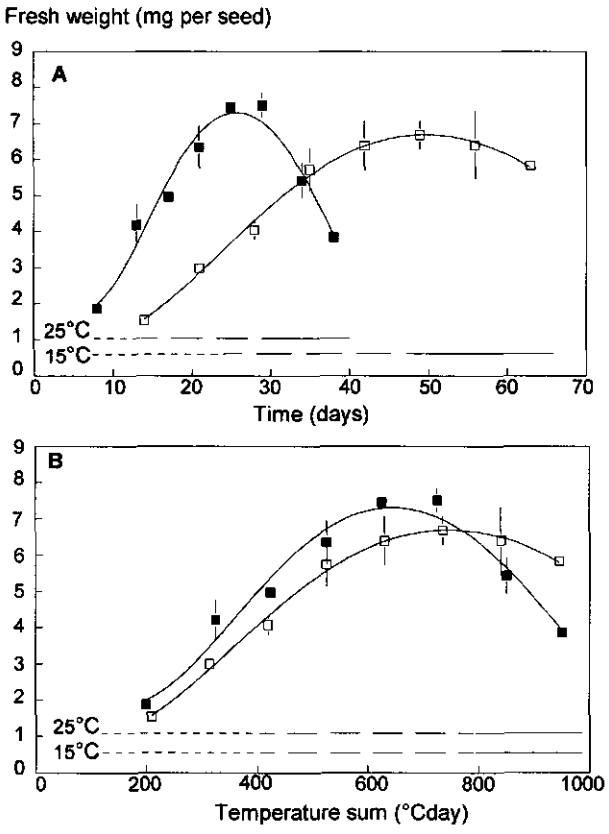


Figure 1: Development of seeds based on days after anthesis (A) and on temperature sum (B). □: 15°C, ■: 25°C. Data are means ± SE (vertical bars, n=4). Developmental stages are illustrated as: dotted lines: cell division and organogenesis; dashed lines: reserve accumulation; solid lines: maturation.

Dissection of single seeds led to a set of physiologically distinct stages for zygotic embryos in seeds (table 1). Morphological stages, at least up to mid-cotyledonary embryos, are reached at the same temperature sums for both

microspore-derived and zygotic embryos. MDEs did not reach the later maturation stages, probably due to their aqueous environment. A base temperature of 0 °C, giving the best fit for available data, indicates that rapeseed becomes physiologically active from this temperature upwards.

Fatty acid accumulation during seed development.

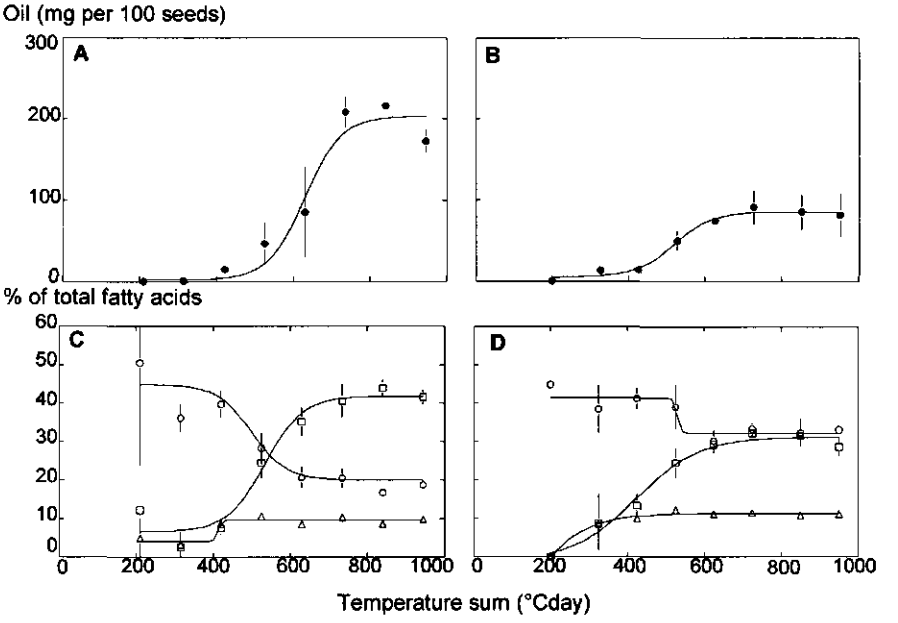


Figure 2: Total oil accumulation (A,B) and relative amounts of the main monounsaturated fatty acids in mol% of total fatty acids (C,D) in developing seeds of Reston.
A,C: 15°C. B,D: 25°C. Fatty acids: 18:1 (○); 20:1 (Δ); 22:1 (□). Data are from a single experiment and represent means ± SE (vertical bars, n=2).

Experiments were performed in two consecutive years. All three cultivars accumulated substantial amounts of seed oil, as is shown for the cultivar Reston (fig 2A/B). Differences between years were observed for total oil accumulation which might be due to light conditions in the greenhouse during development.

Table 2: Fatty acid composition and total oil accumulation of mature seeds of Aurora, Gulle and Reston grown at 15 or 25°C. Data are means ± SE (n=4).

	Fatty acid composition (mol%)[1]							Ratio	Total amount
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	22:1/18:1	(mg)[2]
Aurora 15°C	5.4 ± 0.2	1.7 ± 0.1	67.5 ± 1.1	15.9 ± 0.8	8.3 ± 0.7	0.9 ± 0.1	n.d.[3]	-[3]	143.2 ± 15.3
25°C	5.4 ± 0.3	1.9 ± 0.2	71.3 ± 1.6	16.8 ± 1.2	4.2 ± 0.7	0.5 ± 0.2	n.d.	-	139.3 ± 6.9
Reston 15°C	4.5 ± 0.2	1.0 ± 0.0	21.0 ± 2.0	13.8 ± 0.2	9.7 ±0.6	10.5 ± 0.5	39.5 ± 2.1	2.0 ± 0.3	263.2 ± 59.7
25°C	4.8 ± 0.1	1.3 ± 0.5	36.5 ± 2.4	14.3 ± 1.3	3.2 ± 1.2	12.3 ± 0.7	27.7 ± 1.1	0.8 ± 0.1	89.4 ± 11.8
Gulle 15°C	4.7 ± 0.2	0.7 ± 0.1	15.6 ± 1.1	14.3 ± 1.2	11.9 ± 0.4	9.3 ± 0.4	43.3 ± 1.6	2.8 ± 0.3	228.9 ± 44.3
25°C	5.2 ± 0.2	0.8 ± 0.1	20.2 ± 2.2	16.0 ± 1.1	8.6 ± 0.7	9.3 ± 1.1	39.9 ±1.7	2.1 ± 0.4	114.8 ± 4.5

[1] Trace amounts of 16:1, 20:0 and 22:0 omitted from calculations. [2] Total amount: mg triglycerides per 100seeds, [3] n.d.: not detectable, -: not calculated.

Table 3: Fatty acid composition and total oil accumulation of microspore-derived embryos of Aurora, Gulle and Reston grown at 15 or 25°C at ±1000 °Cday. Data are means ± SE (n=4).

	Fatty acid composition (mol%)[1]							Ratio	Total amount
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	22:1/18:1	(mg)[2]
Aurora 15°C	3.5 ± 2.7	0.5 ± 0.5	82.3 ± 10.5	9.9 ± 5.8	3.8 ± 2.2	n.d.[3]	n.d.	-[3]	6.5 ± 4.4
25°C	1.6 ± 1.9	n.d.	91.5 ± 6.9	5.9 ± 4.6	1.1 ± 1.3	n.d.	n.d.	-	2.7 ± 0.9
Reston 15°C	9.8 ± 0.4	4.1 ± 0.4	41.3 ± 6.8	10.6 ± 6.8	4.6 ± 1.2	10.7 ± 0.1	18.6 ± 4.3	0.5 ± 0.1	16.7 ± 6.9
25°C	6.4 ± 0.4	2.8 ± 0.8	55.5 ± 5.9	6.6 ± 0.8	2.1 ± 0.6	13.5 ± 2.1	13.1 ± 3.9	0.2 ± 0.1	21.5 ± 10.2
Gulle 15°C	6.1 ± 0.3	1.9 ± 0.3	37.2 ± 0.2	9.6 ± 1.9	3.5 ± 1.0	15.0 ± 0.9	26.7 ± 1.4	0.7 ± 0.0	70.7 ± 8.4
25°C	7.4 ± 0.1	n.d.	39.9 ± 5.0	9.5 ± 0.3	1.9 ± 2.7	14.7 ± 1.7	26.7 ± 3.8	0.7± 0.2	41.6 ± 5.6

[1] Trace amounts of 16:1, 20:0 and 22:0 omitted from calculations. [2] Total amount: mg triglycerides per 100 embryos. [3] n.d.: not detectable, -: not calculated.

In Aurora no erucic acid could be detected (table 2), as was to be expected. In comparison with oil from seeds grown at 15 °C the oil from mature seeds grown at 25 °C contained a somewhat lower level of linolenic acid (18:3) which was compensated by an increase in oleic acid (18:1). The levels of other fatty acids were not affected by temperature, nor was the total amount of seed oil.

In Reston accumulation of oil mainly took place between 400 and 700 °C·day leading to over 200 mg oil per 100 seeds at 15 °C and to about 90 mg per 100 seeds at 25 °C (fig 2 A/B, table 2). Considerable amounts of erucic acid (22:1) accumulated at both temperatures (table 2). In this cultivar the levels of 18:3 and 22:1 were higher at 15 °C than at 25 °C, while 18:1 was lower. Early in development 18:1 is the predominant fatty acid (fig 2 C/D) but at about 500 °C·day 22:1 accumulated at a high rate leading to the final, stable level in mature seeds beyond about 700 °C·day.

For developing Gulle seeds the time course of oil accumulation was similar to that observed for developing Reston seeds (data not shown), except for the fact that in Gulle the accumulation of erucic acid was much less affected by temperature. Both at 15 and 25 °C about 40% of fatty acids in the oil was erucic acid (table 2).

Effects of temperature switch during seed development.

When plants of the high erucic acid-type Reston were transferred from 15 to 25°C after more than 500 °C·day the oil showed a high 22:1 content, similar to seeds that developed entirely at 15°C, *i.e.* the temperature change had little effect at this stage of development (fig 3A, open symbols, right part). Seeds from plants transferred to 25°C prior to 500 °C·day had a lower level of 22:1 (fig 3A, open symbols, left part). Similarly, when seeds set at 25 °C were transferred to 15 °C at an early stage they produced seed oil with high levels of 22:1, while seeds on plants exposed to 15 °C late in development produced oil with lower 22:1 levels (fig 3A, closed symbols).

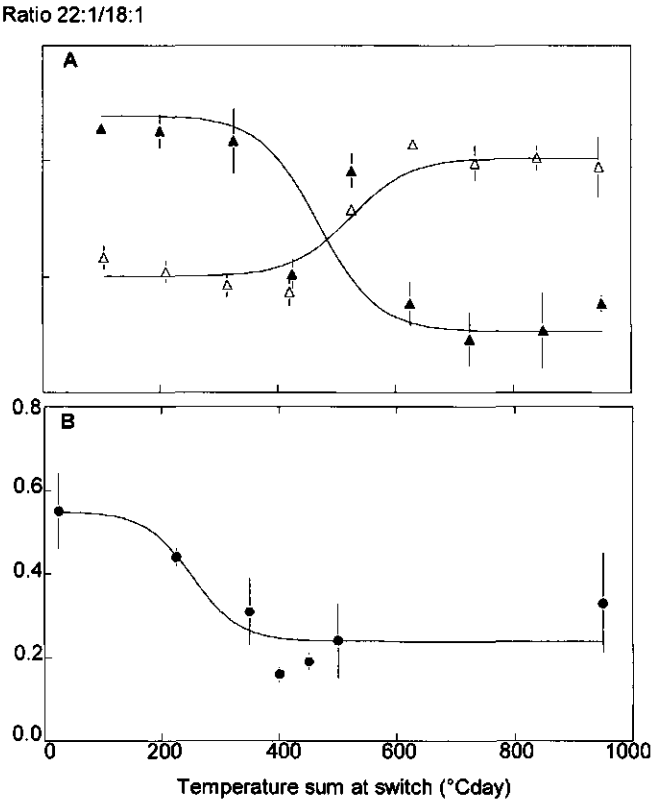


Figure 3: Levels of erucic acid in the oil of mature seeds (A) and microspore-derived embryos (B) of Reston after temperature switch treatment.
Δ: start temperature 15°C; Δ,●: start temperature 25°C. Data are from a single experiment and represent means ± SE (vertical bars, n=4).

In seeds of Gulle these effects are much less pronounced. Changes in fatty acid composition can completely be accounted for by small fluctuations in 18:1 and 18:3, without any effect on the 22:1 level (data not shown).

Development and oil production in microspore-derived embryos.

For rapeseed cultivars Aurora, Gulle and Reston optimum yields of MDEs were obtained with microspores from flower buds of 3.4 to 3.6 mm (data not shown). These embryos showed a morphological development similar to seeds as listed in table 1 during the first 500-600 °C·day. After this stage zygotic embryos are

restricted in their expansion by the surrounding fruit tissues and after about 800 °C·day maturation phenomena like browning and desiccation become visible.

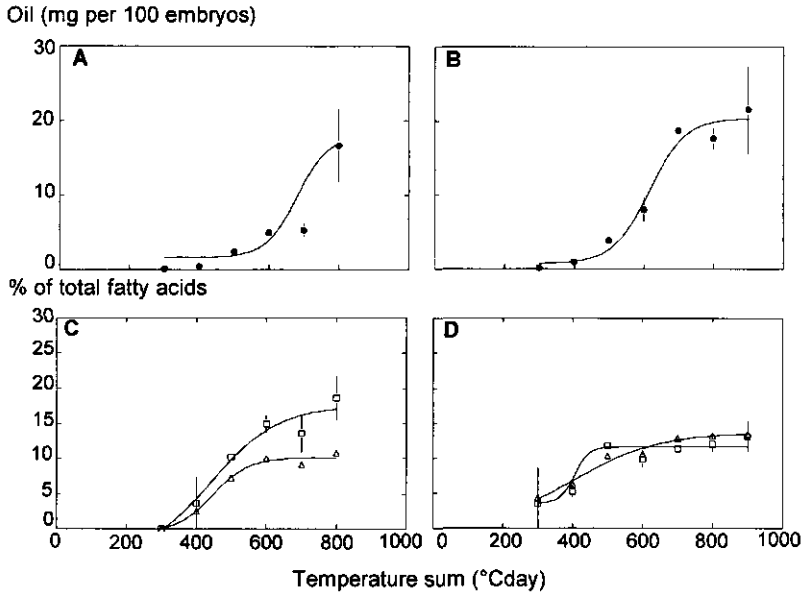


Figure 4: Total oil accumulation (A,B) and relative amounts of very long chain monounsaturated fatty acids in mol% of total fatty acids (C,D) in a typical experiment with developing MDEs of Reston.
A,C: 15°C. B,D: 25°C. Fatty acids: 20:1 (Δ); 22:1 (□). Data are means ± SE (vertical bars, n=2).

In MDEs the accumulation pattern of oil parallels that in seeds at least up to 700 °C·day as is shown for Reston (figs 2 and 4). Morphological features of later maturation stages as in seeds are not visible, which makes it difficult to determine the precise developmental stage. To investigate whether accumulation of oil and changes in its fatty acid composition continue after about 900 °C·day, embryos were harvested with 100 °C·day intervals up to 1400 °C·day in a separate experiment. Determination of the total oil accumulation and the fatty acid composition of the oil showed that after 800 °C·day little further accumulation takes place and that the oil composition is stable (not shown).

A comparison was made of the fatty acid composition of oil from MDEs developed at 15 and 25 °C. Under the conditions tested Aurora embryos produced small amounts of oil with a very high 18:1 content, while levels of linoleic acid (18:2) and 18:3 were low and no eicosenoic acid (20:1) or 22:1 could be detected in any of the samples (table 3). In oil from Gulle and Reston embryos levels of 18:2 and 18:3 were also reduced and 20:1 was increased in Gulle in comparison with seeds (compare tables 2 and 3). Levels of 20:1 in oil from MDEs were in the range of 11 to 15 mol%, but the levels of 22:1 did not exceed 19 mol% and 27 mol% in Reston and Gulle (table 3), respectively, while at the same time 18:1 exceeded 40 mol% in Reston. Levels of 22:1 in oil of MDEs from Gulle were similar at 15 °C and 25 °C, while in MDEs from Reston 22:1 levels were higher at 15 °C than at 25 °C, as was found in seeds.

The considerable difference in total oil production per embryo between Reston and Gulle (table 3) is related to a lower frequency of embryogenesis and concomitant larger size of the individual embryos in Gulle. For a number of samples of mature embryos oil production was calculated on a dry weight basis and then no differences could be found for either cultivars or sizes of mature embryos (data not shown).

When MDEs from Reston and Gulle were exposed to a temperature switch at developmental stages similar to that described for seeds, only MDEs from Reston showed a response comparable to that in seeds (fig 3B). The level of 22:1 was already fully determined at 400 °C·day, while in seeds determination took place after more than 500 °C·day. In these experiments only the temperature sequence 25 °C followed by 15 °C was tested because at 15 °C the early stages of embryogenesis proceed slowly and asynchronously, resulting in populations that were too heterogeneous for evaluation of temperature sensitivity at those stages.

2.5 Discussion

Temperature sum as a measure of developmental stage.

In previous studies developmental age of seeds has been described in terms of days after flowering, which is a good approach when plants are grown at a single temperature. This measure may lead to confusion when processes occurring at different temperatures are compared (fig 1). Therefore developmental processes were linked to temperature sum in this study. Seeds and MDEs, grown at either 15 °C or 25 °C, accumulate oil and 22:1 herein around 500 °C·day and mature at the same rate in about 900 to 1000 °C·day. A slower accumulation of 22:1 in oil of MDEs at 15 °C than at 25 °C, described by Albrecht *et al.* (1994), can thus be explained as a consequence of the slower rate of development at the lower temperature. These data indicate that the use of temperature sum as a measure of developmental stage allows a valid comparison between these two systems.

Genotypic differences in temperature sensitivity and regulation of 22:1 levels.

When we compare oil accumulation in seeds of the cultivars Gulle and Reston for various treatments it is evident that both cultivars are not equally sensitive to growth temperature with respect to 22:1 accumulation. The increase of 22:1 levels at lower temperature in Reston is in agreement with similar observations with cv. Nugget (Canvin, 1965). The sum of monounsaturated fatty acids in the oil is about 70 mol% of total fatty acids at both 15 °C and 25 °C, while no increase in polyunsaturated fatty acids is found parallel to increases in 18:1, not even when comparing Reston and Gulle to Aurora. Downey & Craig (1964) already described similar effects on monounsaturated fatty acids at the level of cultivars. The level of 20:1 is stable at different growth temperatures in both high erucic acid cultivars. Taken together this implies that the elongation from 18:1 to 22:1 and desaturation of fatty acids are controlled independently and that 18:1 levels do not determine either activity.

The final level of 22:1 in Reston may be regulated at different stages during embryo development resulting in different patterns of changes in the level of 22:1 in response to a temperature change. If developing Reston embryos are predisposed early in development to accumulate a certain level of 22:1 the shift in 22:1 levels should be related to temperature switches before maximum oil accumulation occurs. If preformed oil can be modified, *e.g.* by transacylating enzymes, the shift in 22:1 levels will occur with a temperature shift late in development, after the time of maximum oil synthesis. With this model in mind our data strongly suggest that neither predisposition nor modification of oil is involved in seeds of Reston. The shift from high to low erucic acid and *vice versa* occurs very close to 500 °C·day (fig 3A), concomitant with maximum erucic acid synthesis (fig 2C/D). For Gulle on the other hand there is no response to temperature with respect to 22:1 levels. Therefore no sensitive period can be determined.

Microspore-derived versus zygotic embryos.

In MDEs the amount of oil produced per unit (seed or embryo) is lower than in seeds (tables 2 & 3) mainly due to a reduction in unit dry weight, when calculations are made on a percentage of dry weight basis the differences are much smaller. All embryos reached a similar morphological stage before harvest. In Aurora this led to MDEs with a high level of 18:1 in the oil. This is in contrast to conclusions from previous results with cultivars Topas and Reston describing that seeds and MDEs behave similarly with regard to quality and per unit quantity of oil synthesis (Taylor *et al.*, 1990, 1991, Taylor & Weber, 1994). In Aurora traces of 20:1 as found in seeds could not be detected in MDEs, probably due to lower oil synthesis resulting in a lack of substrate for the low elongase activity present.

In Reston and Gulle the fatty acid distribution shows changes in polyunsaturated fatty acids similar to Aurora, but elongase activity is present in

the MDEs from the former two cultivars as can be seen from the presence of 20:1 and 22:1. Levels of 20:1 are similar to those in seeds, but 22:1 is reduced considerably, especially in Reston. This is an indication that despite broad similarities at least quantitative differences occur between MDEs and seeds with respect to oil biosynthesis. When data from the temperature switch experiments with seeds and MDEs are compared it is clear that some factor regulating the level of 22:1 has changed, not only in its quantitative effects but also in the timing of the regulation by temperature, resulting in a shift of the sensitive period from 500 to 300 °C·day.

A possible candidate for this missing factor is abscisic acid (ABA). Finkelstein *et al.* (1985) found that levels of ABA of embryos cultured in vitro are reduced threefold below the basal level found in embryos freshly prepared from developing seeds. Addition of ABA to cultures of MDEs of rapeseed resulted in increased levels of 22:1 (Albrecht *et al.*, 1994) due to stimulated synthesis of 22:1 (Holbrook *et al.*, 1992). Also mutants of *Arabidopsis thaliana* with respect to ABA-synthesis and -sensitivity were reported to have reduced levels of 20:1, the predominant very long chain fatty acid in this species (de Bruijn, 1993). We are currently investigating a possible interaction between temperature response and ABA levels in relation to the fatty acid composition of seed oil in *Brassica napus* L.

Acknowledgements

We like to thank Dr. R. Scarth at the Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada, for the gift of Reston Seeds and Dr. L. Bengtsson of Svalöf AB, Svalöv, Sweden, for the gift of the Gulle-derived line.

Chapter 3

EFFECTS OF ABSCISIC ACID AND TEMPERATURE ON ERUCIC ACID ACCUMULATION IN OILSEED RAPE (*Brassica napus* L.)

Jeroen A. Wilmer, Johannes P.F.G. Helsper and Linus H.W. van der Plas

This chapter was published in: J. Plant Physiol. 150: 414-420 (1997)

Keywords

Brassica napus L.; abscisic acid; ABA; erucic acid; microspore-derived embryos; oilseeed rape; seeds; temperature

3.1 Summary

The qualitative effect of growth temperature on the level of erucic acid (22:1) is similar in seeds and microspore-derived embryos (MDEs) of oilseed rape, as we have previously shown (chapter 2), but absolute levels of 22:1 are lower in MDEs. In this paper we studied whether this temperature effect is mediated by changes in the level of or sensitivity to abscisic acid (ABA).

For this purpose we measured endogenous levels of ABA in developing seeds and MDEs. In addition, 22:1 accumulation was determined in MDEs exposed to a range of concentrations of ABA. The endogenous level of ABA in seeds, about 5 to 10 nmol/g FW, was twentyfold higher than the concentration at which maximum sensitivity in MDEs occurred. Further evaluation using dose-response curves showed that at both 15 and 25 °C the half-maximum increase in 22:1 level in MDEs was observed at about 0.3 µmol/l ABA, though throughout the concentration range tested levels of 22:1 were about 10 mol% higher at 15 °C than at 25 °C. This indicates that the sensitivity to ABA remains the same in this temperature range. Independent from the temperature effect, exogenously applied ABA induces an increase of about 10 mol% in 22:1 within the range of 10 to 25 °C. In addition to this effect on 22:1 level, ABA stimulates the production of triglycerides in MDEs, whereas temperature has no effect on oil accumulation. In combination our data indicate that growth temperature and ABA are independent and additive stimuli in regulating the level of 22:1 in oilseed rape.

3.2 Introduction

The plant growth regulator abscisic acid (ABA) has been implicated in a number of processes occurring during seed development. They include regulation of the accumulation of storage products like proteins (White & Rivin, 1993, Pomeroy *et al.* 1994, Xu & Bewley, 1995) and lipids (Finkelstein & Somerville, 1989). Based on the latter role, ABA is of interest in our studies on the regulation of erucic acid (22:1) accumulation in oil of oilseed rape. Holbrook *et al.* (1992) reported that addition of ABA to microspore-derived embryos (MDEs) relatively early in development resulted in increased levels of erucic acid which was associated with an increase in elongase activity.

In a previous study we found that levels of 22:1 are lower in MDEs than in zygotic embryos in seeds of oilseed rape (chapter 2), although development of these two types of embryos was similar in most aspects (Taylor *et al.*, 1990, Chen & Beversdorf, 1991, Albrecht *et al.*, 1994). An increase of 22:1 levels with decreasing temperature was observed in MDEs as described for seeds by Canvin (1965), although the total level of 22:1 was lower in MDEs. One major difference between seeds and MDEs is the potential presence of signalling molecules produced by maternal tissue in seeds, the latter being absent in MDEs. This difference and the effects of ABA described above suggest that ABA from either maternal origin or from the embryos is a factor regulating 22:1 levels and possibly mediating the effect of temperature. If maternal ABA is important, the lack of maternal tissue in MDEs may explain the reduction in 22:1 levels. If embryonic ABA is important, leakage of this ABA into the surrounding medium could explain the reduced levels of 22:1 in MDEs. Finkelstein *et al.* (1985) already showed a considerable reduction in endogenous ABA levels after 3 days in embryos isolated from seeds.

In this study we measured 22:1 levels in the oil from MDEs grown at different temperatures and at different levels of exogenous ABA to determine whether ABA plays a role in mediating the effect of temperature. We also compared

levels of ABA in seeds and MDEs on one hand and sensitivity to ABA in MDEs on the other hand. The possible role of ABA as the factor responsible for the observed differences in erucic acid content in seeds and MDEs is discussed.

3.3 Materials & methods

Plant material.

Two high erucic acid genotypes of spring rape were used in our experiments: Reston, from the University of Manitoba, Canada, and a selection from cv. Gulle, obtained from Svalöf AB, Sweden (further referred to as "Gulle"). Plants were grown in the greenhouse in potting soil in 2-litre pots, at 20/17 °C (day/night) at a photo period extended to 16 hours with SON-T AGRO (Philips) to ensure induction of flowering. At the onset of flowering racemes were harvested for the induction of microspore-derived embryos. For determination of *in vivo* ABA levels, seeds were harvested at different stages, defined by temperature sum reached after anthesis (chapter 2).

Culture of microspore-derived embryos.

MDE culture was performed according to Wilmer *et al.* (chapter 2). Briefly, racemes were collected at the onset of flowering and buds of 3.4 to 3.6 mm length were selected. The buds were sterilised in 2% NaClO and homogenised in a modified Lichter medium (NLN13). After filtration microspores were collected by centrifugation, washed, diluted to 40000 spores ml^{-1} and 1 ml portions of suspension were plated in 35 mm Petri dishes. Embryogenesis was induced at 32 °C, then cultures were transferred to 25 °C for another 4 days (total temperature sum: 200 °C·day). At this point embryos for 10, 15 and 20 °C-treatments were transferred to that temperature. ABA-treatments were started at 350 °C·day by replating the embryos in fresh medium containing the desired concentration of ABA freshly diluted from 0.1 M (±)-ABA in methanol, controls were replated in medium containing 0.1‰ methanol. Concentrations of

ABA given in the text refer to effective concentrations of only (+)-ABA. After reaching a temperature sum of 800 °C·day embryos were rinsed with tap water and stored at -80 °C for analysis of the oil composition.

Lipid extraction.

Embryos (50-300 mg) were homogenised using an Omni 2000 homogeniser with a 1 cm-probe in 5 ml of a mixture of methanol : chloroform : 0.01 N hydrochloric acid (2:1:0.8, by vol.), containing trimargarin as an internal standard. After shaking for 15 min the extract was transferred to a screw-capped centrifuge tube and chloroform and 0.01 N hydrochloric acid were added to obtain a mixture with a ratio 2:2:1.8 by volume. After thorough mixing the suspension was centrifuged to achieve phase separation. The aqueous phase was washed once with 1.5 ml synthetic organic phase, organic fractions were combined and stored under nitrogen at 4 °C until further analysis.

Thin-layer chromatography and transesterification of lipids.

Aliquots of chloroform-extracts were applied to 20 x 20 cm TLC plates which were developed in hexane : diethyl ether : acetic acid (70:30:1, by vol.). The plates were dried and lipids visualised with iodine vapour. Areas containing triglycerides were scraped off and the powder transferred to 3 ml 5% sulphuric acid in methanol. After flushing with nitrogen, tubes were capped and incubated at 70 °C for three hours, with shaking every 15 min during the first 1.5 hours. Subsequently, 3 ml of water and 3 ml of hexane were added and mixed by rotation for 15 min to extract the fatty acid methyl esters (FAMEs) into the organic layer. After centrifugation the organic layer was collected and the methanol/water mixture was washed with 1.5 ml hexane. The two organic fractions were combined and stored at 4°C for GC-analysis.

GC-analyses.

FAMEs were analysed using a capillary Chrompack CP9000 gas-chromatograph equipped with a 25 m x 0.32 mm CPwax 52 CB column and flame ionisation detection. The injector temperature was 260 °C, the detector temperature 300 °C, the oven temperature was programmed from 190 °C (4.1 min) to 230 °C (4.4 min) at 20 °C min^{-1}. Helium was used as carrier gas at 80 kPa. FAMEs were identified on the basis of retention time and quantified by electronic integration with reference to methyl margarate, derived from the internal standard trimargarin.

ABA extraction and determination

ABA extraction and purification were performed essentially as described in Kim *et al.* (1994). Embryos (20-100 mg) or seeds (100-300 mg) were frozen and homogenised in a mixture of methanol: ethyl acetate: water (3:3:2, by vol.) containing 100 mg/l tert-butyl hydroxytoluene. The mixture was centrifuged for 10 min at 2000 x g and the pellet washed with the same medium. The combined supernatants were passed through two consecutive Sep-Pak C_{18} cartridges (Millipore). After elution the ABA-extract was evaporated to dryness and the residue redissolved in 0.5 ml PBS-Tween (Ross *et al.*, 1987).The amount of ABA was estimated by an enzyme immunoassay as described for cytokinins (Vonk *et al.*, 1986), using ABA-oxime coupled to keyhole limpet hemocyanin and a monoclonal antibody purchased from IDETEK Inc. (Ross *et al.*, 1987).

Chemicals

All solvents used were HPLC or reagent grade. Trimargarin, ABA and FAME-standards were purchased from Sigma and TLC-plates (0.5 mm Silica gel 60 plates) from Merck.

Statistics

Pairwise comparisons of sets of ABA-levels and 22:1 levels were done using Student's t-test (α=0.05). Analysis of interaction between temperature and ABA was performed using a two-factor ANOVA (α=0.05).

3.4 Results

ABA levels in developing seeds and MDEs

Table 1: Levels of endogenous abscisic acid in developing seeds in nmol/g FW and 22:1 levels at seed maturity in cv. Reston and Gulle. Data are means ± SE of duplicate samples.

		Maximum level at 420 °C·day	Basal level at 850 °C·day	22:1 at maturity (% of total fatty acids)
Reston	25 °C	3.5 ± 1.5	1.2 ± 0.1	27.7 ± 1.1
	15 °C	6.4 ± 1.6	1.2 ± 0.1	39.5 ± 2.1
Gulle	25 °C	1.6 ± 1.4	0.5 ± 0.1	39.9 ± 1.7
	15 °C	11.9 ± 1.9	1.2 ± 0.5	43.3 ± 1.6

In seeds of both Gulle and Reston the peak levels of ABA, reached at about 400 °C·day (early cotyledonary stage), were higher at 15 °C than at 25 °C (table 1). In Gulle this difference in ABA-levels was much larger than in Reston. Final levels of ABA at 850 °C·day (almost mature seeds) were low and similar for both cultivars (table 1). In MDEs about 0.4 nmol ABA per gram fresh weight was measured and levels decrease even further to about 0.02 nmol/g after transfer to fresh media without ABA (data not shown).

Table 2: Levels of endogenous abscisic acid (ABA) in MDEs of Reston after exposure to 5 µM (+)-ABA or control medium for 12h and erucic acid at maturity. Data are means ± SE of triplicate samples.

		ABA level (nmol/g FW)	22:1 at maturity (% of total fatty acids)
25 °C	+ ABA	11.1 ± 3.5	32.2 ± 1.4
	control	0.12 ± 0.02	21.7 ± 0.1
15 °C	+ ABA	17.8 ± 2.2	30.0 ± 2.6
	control	0.08 ± 0.04	20.1 ± 1.3

In a short-term experiment MDEs of Reston, treated with 5 µmol/l (+)-ABA, accumulated endogenous ABA levels that increased sharply within the first hour, reaching a maximum of 11-18 nmol/g fresh weight after about 12 h (fig 1).This is about hundredfold the concentration found in non-treated MDEs (table 2). After 48 h ABA levels had decreased again but levels were still higher than those in non-treated embryos. Addition of ABA resulted in an increase in 22:1 levels at maturity (800 °C·day) from 20 to 30 mol% of total fatty acids both at 15 and 25 °C in this experiment (table 2).

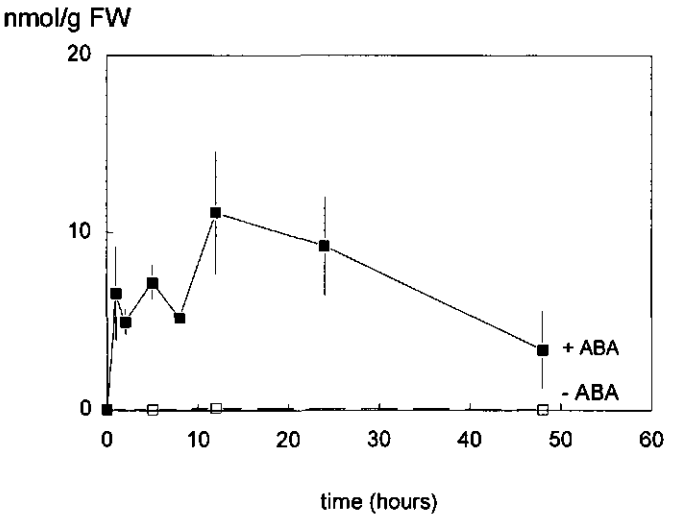


Fig. 1: Levels of endogenous ABA in MDEs of Reston cultured in medium containing 5 µM (+)-ABA (■) or in medium without ABA (□). Data are means ± SE (vertical bars) of triplicate samples.

In MDEs grown for 3 days or more on medium containing up to 50 µmol/l ABA the endogenous level of ABA was 0.05 - 0.1 nmol/g (data not shown). This level is slightly higher than found in control treatments but still lower than in MDEs before transfer to fresh medium. In MDEs of Gulle similar effects were found (data not shown).

Effects of ABA and temperature in MDEs

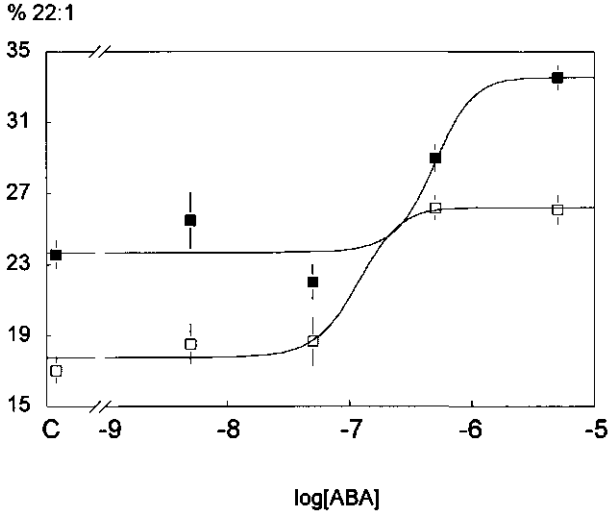


Fig. 2: Effect of adding different concentrations of (+)-ABA at 350 °C·day on the level of 22:1 at 800 °C·day in MDEs of Reston at 25 °C (□) and 15 °C (■). Data are means ± SE (vertical bars, n = 5)

Dose-response curves (fig 2) show that both at 15 and 25 °C, the 22:1 level in response to exogenous ABA reaches its maximum at medium concentrations between 0.5 and 1 µmol/l ABA. Concentrations below 0.05 µmol/l are no longer effective. In a number of separate experiments the ABA concentration evoking a half maximum response ($[ABA]_{50}$) was estimated at 0.41 ± 0.12 µmol/l and 0.26 ± 0.19 µmol/l for MDEs grown at 15 and 25 °C, respectively. This lack of a significant difference indicates that the affinity to ABA does not change with growth temperature and hence cannot have been the cause of differences in the 22:1 level. Addition of ABA not only increased the level of 22:1, which was compensated by a decrease in 18:1, but it also induced a small increase in linolenic acid (18:3; Möllers *et al.*, 1994) and an increase in total oil (table 3).

Table 3: Fatty acid composition and total oil accumulation in microspore-derived embryos of Reston grown at 15 or 25°C in the presence or absence of 5 µM (+)-ABA. Data are means ± SE (n=5).

	Fatty acid composition (mol%)[1]							Total amount
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	(mg)[2]
25°C - ABA	5.0 ± 0.1	2.0 ± 0.1	47.6 ± 1.0	10.8 ± 0.2	4.0 ± 0.2	13.5 ± 0.3	17.0 ± 0.8	10.1 ± 0.1
+ ABA	5.6 ± 0.1	1.9 ± 0.1	38.4 ± 1.2	11.2 ± 0.7	5.0 ± 0.6	11.8 ± 0.4	26.1 ± 0.9	16.0 ± 0.8
15°C - ABA	6.9 ± 0.2	2.2 ± 0.6	35.2 ± 1.6	15.1 ± 0.6	8.0 ± 0.8	9.1 ± 0.7	23.7 ± 0.8	8.0 ± 0.7
+ ABA	4.8 ± 0.5	1.5 ± 0.4	26.3 ± 0.6	14.4 ± 0.5	10.1 ± 0.4	9.6 ± 0.2	33.5 ± 0.7	17.6 ± 0.3

[1] Trace amounts of 16:1, 20:0 and 22:0 are omitted from calculations. [2] Total amount: mg triglycerides per 100 embryos.

The increase in 22:1 at lower temperature was not only accompanied by a decrease in 18:1, but also by increases in linoleic acid (18:2) and 18:3 (Tremolières et al., 1982, chapter 2). Temperature had small and inconsistent effects on total oil accumulation (table 3).

At incubation temperatures between 10 and 25 °C, addition of ABA at a saturating concentration of 5 µmol/l in the culture medium resulted in a 7 to 10 mol% increase of the 22:1 level (fig 3). The resulting 22:1 levels are comparable with those found in mature Reston seeds (table 1). Analysis of variation revealed no interaction between ABA and growth temperature ($p \sim 0.13$).

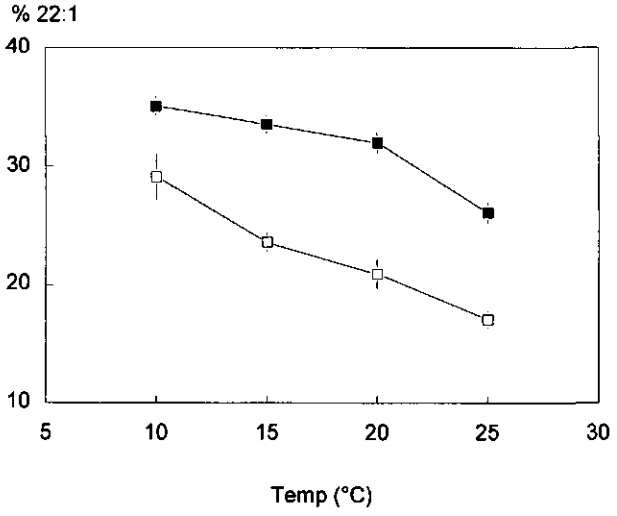


Fig. 3: Effects on the level of 22:1 in mature MDEs of Reston of different temperatures in combination with 0 (□) and 5 µM (+)-ABA (■) added at 350 °C·day. Data are means ± SE (vertical bars, n = 5)

3.5 Discussion

The results presented in this study show that both temperature and ABA affect 22:1 levels in the oil from MDEs. If ABA is an intermediate in the temperature effect on 22:1 content it might act at two levels in the signalling pathway:

temperature may affect either the endogenous ABA concentration or the sensitivity to this plant growth substance.

If the effect of growth temperature on the level of 22:1 is only mediated by ABA concentration in developing embryos (seeds or MDEs) without any effects of changes in sensitivity, the changes in the levels of ABA in these tissues have to fulfil certain requirements. First, as addition of ABA increases the level of 22:1 in MDEs (Holbrook *et al.*, 1992), levels of ABA should be higher in seeds grown at 15 °C than in seeds grown at 25 °C. This is in agreement with our data (table 1). However, as differences in 22:1 are larger in seeds of Reston than in seeds of Gulle, the differences in ABA levels in Reston are expected to be larger than in Gulle. The latter is not in accordance with the data, although it might also be explained by genotypic differences in sensitivity. A second important factor is that levels of ABA should be in the range of maximum sensitivity to this growth regulator. Determination of ABA-sensitivity in seeds by varying the levels of ABA is difficult as very high concentrations of ABA are necessary to reach a significant increase over endogenous levels of 5 to 10 nmol/g FW, equivalent to concentrations of about 10 µmol/l (table 1). Since suitable ABA-synthesis mutants in oilseed rape are not available reduction of ABA requires addition of chemicals like fluridone. However, the use of fluridone, a general inhibitor of carotenoid synthesis (Bramley, 1994), will lead to undesired side effects including damage to the photosynthetic machinery within the siliques, which is required for dry matter accumulation in the seeds of oilseed rape and related species (Singal *et al.*, 1995). Thus, at present manipulating ABA levels in seeds of *B. napus* does not seem to be a feasible approach.

In *Arabidopsis thaliana* mutants in both ABA synthesis (*aba*) and ABA perception (*abi*; Koornneef *et al.*, 1984) are available and they have been used in a number of studies. In these studies levels of 20:1 were about 20% in wildtype seeds and about 7% in an ABA insensitive mutant *abi3-1*. De Bruijn (1993) found that the level of 20:1 in the ABA synthesis mutant *aba1* was

reduced to about 7% whereas Finkelstein and Somerville (1990) found that the same line contained wildtype levels of 20:1. In this *aba1* mutant the ABA content is reduced to a few percent of the wildtype level and the results of de Bruijn suggest that ABA may become a limiting factor in 20:1 synthesis. This is an indication that wildtype levels of ABA are higher than required for saturation of the regulatory mechanism for elongase activity.

To further study the role of changes in ABA tissue concentrations in oilseed rape, we used MDEs as a system which contains less endogenous ABA, but is responsive to ABA. MDEs retain sensitivity to temperature (chapter 2), and ABA levels in the medium can easily be manipulated. The dose-response curve (fig 2) shows that maximum sensitivity, defined by $[ABA]_{50}$, in MDEs is reached at about 0.3 to 0.4 µmol/l, which is about twenty times less than endogenous levels in seeds accumulating oil (table 1). Although it can be explained by differences in the distribution of ABA in the tissue in seeds and MDEs, this result does not support the hypothesis that the effect of temperature on 22:1 in seeds is mediated by a change in ABA concentration. Only if the affinity to ABA in seeds is about 20- to 50-fold lower than in MDEs does a role of ABA as an intermediate again become possible. Data from the studies on Arabidopsis mutants mentioned above suggest otherwise, as the reduction of ABA levels with a similar factor resulted in highly variable 20:1 levels.

Holbrook *et al.* (1992) found that only the first 3 days were important in inducing an ABA-mediated increase in elongase activity and subsequently in 22:1 level in MDEs. When we tried to correlate exogenously applied levels of ABA with endogenous levels it appeared that this hormone remained present only during a limited time (fig 1). Addition of ABA at concentrations up to 50 µmol/l did not result in increases in endogenous levels after 3 days or more. However, such a treatment was able to induce increased levels of 22:1 at maturity. Assuming about 80% moisture in MDEs at this stage of development it appeared that the application of 5 µmol/l ABA resulted in 3- to 4-fold accumulation over medium-

concentrations in MDEs during the first hours (fig 1, table 2). After about 24 hours these levels started to decrease (fig 1), but the signal may have been transferred by then.

Using MDEs as a model system also allowed us to investigate the other possible form of interaction between temperature and ABA in their effects on 22:1 levels: a change in tissue sensitivity to ABA induced by changes in temperature. Maximum sensitivity, defined as the concentration at which minimum changes in concentration give maximum effects, occurred at about 0.3 µmol/l, well below levels found in seeds (compare table 1 and fig 2) and was not significantly different at 15 and 25 °C (fig 2). Firn (1986) defined this aspect of sensitivity as affinity, and separated it from another component of tissue sensitivity, response capacity, defined in our system as the amplitude of the dose-response curve, the difference between 'control' and maximum response. If we compare the effects of growth temperature on response capacity, again no differences can be found (fig 2); the entire curve shifts downward by about 10 mol% of 22:1 when the growth temperature is changed from 15 to 25 °C.

When we compared the levels of 22:1 in MDEs treated with 5 µmol/l ABA and controls over a temperature range of 10 to 25 °C the difference between ABA-treatments and control remained close to 10 mol% of 22:1; temperature and ABA treatments showed no statistically significant interaction. The lack of interaction or saturation of the temperature response indicate that the effect of temperature is not mediated by a change in ABA concentration or sensitivity to ABA.

Thus, ABA is apparently not an intermediate in the signalling pathway initiated by temperature perception. However, the two factors could influence oil composition using the same transduction chain. But also then some kind of interaction between ABA and growth temperature is to be expected. Moreover, our data show that addition of ABA to the medium results in an increase of 22:1

level of about 10% over the entire temperature range tested, and is accompanied by an increase in total oil production, whereas growth temperature only affects only oil composition, and not the amount of oil in MDEs (table 3). Thus, we postulate that in addition to the fact that ABA is not an intermediate in the transduction of temperature perception to oil composition in this species, the two stimuli also have at least in part different signalling pathways for influencing lipid metabolism.

Acknowledgements

We thank Evert Davelaar for his technical assistance in optimising and performing the ABA-determinations.

Chapter 4

REGULATION OF ELONGASE ACTIVITY BY ABSCISIC ACID AND TEMPERATURE IN MICROSPORE-DERIVED EMBRYOS OF OILSEED RAPE (*Brassica napus* L.)

Jeroen A. Wilmer, René Lessire, Johannes P.F.G. Helsper

and Linus H.W. van der Plas

Keywords

Brassica napus L.; acyltransferase; elongase; erucic acid; microspore-derived embryos; regulation

4.1 Summary

Growth temperature and ABA both affect the level of erucic acid (22:1) in microspore-derived embryos of oilseed rape. We have previously shown that these stimuli act independently (chapter 3). In this study we investigated the effects of growth temperature and ABA on the characteristics and activity of the elongase complex, the enzymes synthesising 22:1. Due to substrate inhibition at low concentrations (<10 µM) of oleoyl-CoA it was not possible to determine values for K_m and V_{max}. Elongase activities from preparations extracted from microspore-derived embryos grown under different conditions showed an optimum temperature higher than 30 °C, with a Q_{10} value of about 3. We found considerable differences in total elongase activity in MDEs grown under different culture conditions, which correlated closely with the absolute amount of 22:1. Our results suggest that the accumulation of 22:1 is regulated by the amount of elongase enzyme and not by changes in the intrinsic characteristics of the enzyme. Correlation between elongase activity and the fraction of 22:1 in oil is poor. Including the total activity of acyltransferases does not improve the correlation, as acyltransferase activity itself correlates poorly with the total amount of oil formed.

4.2 Introduction

In previous investigations it has been shown that the level of erucic acid (22:1) in developing seeds and microspore-derived embryos (MDEs) of oilseed rape is affected by temperature (Canvin, 1965, Albrecht *et al.*, 1994, chapter 2) and abscisic acid (ABA; Holbrook *et al.*, 1992, chapter 3). We have shown that the response to temperature is similar in seeds and MDEs (chapter 2) and that

temperature and ABA act independently in affecting the level of 22:1 (chapter 3). The level of 22:1 reacts to both a decrease in growth temperature from 25 to 15 °C and addition of 5 µM ABA by an increase of about 10 mol% of total fatty acids in the oil. The effects on erucic acid level in the oil have been linked with the activity of enzymes involved in biosynthesis of this fatty acid only in a single study (Holbrook *et al.*, 1992). They showed an increase in elongase activity after treatment with ABA together with an increase in 22:1 level, but tested only one set of assay conditions and a single growth temperature. The effect of temperature on the level of elongase activity has not been documented so far.

In studies on the effect of temperature on fatty acid desaturase activity two hypotheses have been postulated. The first involves the effect of temperature on the availability of oxygen and the fluidity of the membrane. According to this hypothesis lowering the temperature increases the solubility of oxygen and reduces the fluidity of the membrane. Both factors would enhance the activity of the desaturases at lower temperature (Trémolieres *et al.*, 1982). However, elongase reactions do not involve molecular oxygen and therefore a direct control by oxygen is unlikely, but activation of elongase with a lowering of temperature is possible. The second hypothesis involves a differential expression of fatty acid synthase and desaturase genes with temperature, resulting in a higher ratio of desaturase to fatty acid synthase activity with lower temperature. These effects on the activity ratio give rise to the differences in desaturation in the membrane (Browse & Slack, 1983).

In this study we investigated elongase and acyltransferase activities from microspore-derived embryos, grown at two temperatures in the presence or absence of ABA, and determined intrinsic characteristics of the elongase complex. We correlated these data with the total amount of oil accumulated and the accumulation of 22:1 in the oil to explore a possible regulatory mechanism of 22:1 accumulation.

4.3 Materials & methods

Culture of microspore-derived embryos.

Plants of oilseed rape cultivar Reston, obtained from the University of Manitoba, were grown in the greenhouse at 20/17 °C (day/night) at a photo period extended to 16 hours with SON-T AGRO (Philips) to ensure induction of flowering. MDE culture was performed according to chapter 2. Briefly, racemes were collected at the onset of flowering and buds of 3.4 to 3.6 mm length were selected. The buds were sterilised in 2% NaClO and homogenised in a modified Lichter medium (NLN13). After filtration microspores were collected by centrifugation, washed, diluted to 40000 spores ml^{-1} and 1 ml portions of suspension were plated in 35 mm Petri dishes. Embryogenesis was induced at 32 °C, then cultures were transferred to 25 °C for another 4 days (total temperature sum = average day temperature *x* number of days: 200 °C·day). At this point embryos for 15 °C treatments were transferred to that temperature. ABA-treatments were started at 350 °C·day by replating the embryos in fresh medium containing 5µM ABA freshly diluted from 0.1 M (±)-ABA in methanol, controls were replated in medium containing 0.1‰ methanol. The concentration of ABA given refers to the effective concentration of only (+)-ABA. Thus, we made a matrix of 2 temperatures (15 and 25 °C) and 2 ABA treatments (0 and 5µM).

After reaching a temperature sum of 450 °C·day embryos were rinsed with tap water and stored at -80 °C for analysis of elongase activity. Embryos for lipid determination were harvested at 800 °C·day.

Enzyme preparations.

Microspore-derived embryos were ground in a buffer containing 80 mM HEPES, pH 6.8, 0.32 M sucrose, 10 mM ß-mercaptoethanol and 50 mg/ml PVPP at 5 mL buffer per gram tissue. The homogenate was passed through 2 layers of miracloth and centrifuged at 1000 *x* g for 5 min. About 200 µl of the

supernatant was used for the determination of total acyltransferase activity, the remainder was recentrifuged at 15000 *x* g for 25 min to obtain a membrane pellet. The pellet was resuspended in 80 mM HEPES, pH 6.8, 10 mM ß-mercaptoethanol and centrifuged again for 25 min at 15000 *x* g. The amount of protein in the final fractions was determined using Coomassie-Plus (Pierce Inc.) and aliquots were used to assay elongase activity.

Elongase assay.

Elongase was assayed using a procedure modified from the one described by Creach *et al.* (1993), using 15 (exp. 1) or 30 (exp. 2) µg of protein from the 15000 *x* g-pellet, incubated at different temperatures (see results) in a buffer containing 80 mM HEPES, pH 7.2, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$, 150 µM Triton X-100, about 8 µM [2-^{14}C]-malonyl-CoA (1.85 kBq, Amersham) and 0 to 75 µM oleoyl-CoA, in a final volume of 100 µl. After incubation up to 30 min the reaction was stopped by the addition of 100 µl 5N KOH, 10% methanol, and the reaction products saponified for 1.5 h at 70°C. Next 100 µl of 5M H_2SO_4, 10% malonic acid was added and the mixture extracted with 2 ml of chloroform. The organic phase was washed 3 times with 2 ml of water, evaporated under a stream of air and radioactivity was measured in a liquid scintillation counter.

Acyltransferase assay.

Total acyltransferase activity was determined as ^{14}C-incorporation from [U-^{14}C]-glycerol-3-phosphate (G-3-P) using a protocol derived from that described by Taylor *et al.* (1991). Samples containing 25 µg of protein from the 1000 *x* g-supernatant were incubated in a buffer containing 80 mM HEPES, pH 7.2, 2 mM $MgCl_2$, 40 µM oleoyl-CoA, 1 mM ATP and 6.5 µM [U-^{14}C]-G-3-P (3.7 kBq) in a final volume of 100 µl. Incubations were carried out in open tubes at 30 °C for 10 to 30 min. The reaction was stopped by adding 200 µl of 0.01 N

hydrochloric acid and 1 ml chloroform. After thorough mixing the organic and aqueous layers were separated by centrifugation and the organic fraction was washed 3 times with 1 ml water. An aliquot of the organic phase was evaporated to dryness, redissolved in scintillation liquid and radioactivity was determined. The remainder was concentrated under a stream of nitrogen and applied to TLC for analysis of reaction products.

TLC analysis of reaction products from the elongase assay.

Aliquots of fatty acids were methylated using 5% H_2SO_4 in methanol as previously described (chapter 2). After methylation the fatty acid methyl esters (FAMEs) were spotted on reversed phase TLC plates. Plates were developed in acetonitrile : methanol : water 65:35:0.5 (by vol.). After drying FAMEs were visualised and the distribution of radioactivity quantified using phosphor-imaging.

TLC analysis of reaction products from the acyltransferase assay.

Samples were applied to 20 x 20 cm x 0.25 mm Silica gel 60 plates. Plates were developed about two-thirds in methyl acetate : n-propanol : chloroform : methanol : 0.25% aqueous KCl (25:25:28:10:7 by vol.). After drying the plates were fully developed in the same direction in hexane : diethyl ether : acetic acid (70:30:1 by vol.). This results in resolution of all reaction products (Heape *et al.*, 1985). After drying the products were visualised and the distribution of radioactivity quantified using phosphor-imaging.

Lipid extraction and analysis of fatty acid composition

Oil was extracted from mature MDEs and fatty acid composition was determined as fatty acid methyl esters using capillary gas-chromatography as described previously (chapter 2).

Chemicals.

All solvents used were HPLC or reagent grade. Radiochemicals were purchased from Amersham. ABA, buffers, cofactors and FAME-standards were purchased from Sigma and TLC-plates (RV18 F_{254}, cat no. 13724 and Silica gel 60 F_{254}, cat no. 105715) from Merck.

4.4 Results

Reaction conditions.

Addition of either 1 mM ATP or 25 µM 18:1-CoA to a reaction mixture both resulted in about a 70-fold increase in elongase activity determined as ^{14}C-incorporation from [2-^{14}C]-malonyl-CoA over control levels (table 4.1).

Table 4.1: Effects of 18:1-CoA and ATP on elongase activity from MDEs grown at 25 °C and 22:1/20:1 ratio in the resulting products.

	Elongase activity[1]	22:1/20:1
control	0.03	n.d.[2]
+ 1 mM ATP	2.43	0.7
+ 25 µM 18:1-CoA	1.79	0.5
+ ATP, + 18:1-CoA	1.28	0.05

1: Elongase activity in nmol/h·g FW, determined in the presence of 8.5 µM [2-^{14}C]-malonyl-CoA and cofactors.

2: n.d.: not determined

When both ATP and 18:1-CoA were added, elongase activity increased only about 40-fold over the control level with a concomitant strong decrease in the ratio of [^{14}C]-22:1 to [^{14}C]-20:1. Therefore, only 18:1-CoA and [2-^{14}C]-malonyl-CoA were added as substrates and ATP was omitted in all further determinations.

18:1-CoA and temperature dependence of the elongase complex.

Elongase activity was determined, using 18:1-CoA in a range of concentrations (fig 4.1). In vitro elongase activity reached a maximum at approximately 10 µM

18:1-CoA for enzyme preparations from MDEs grown in the different conditions tested. At higher concentrations the activity decreased indicating substrate inhibition by 18:1-CoA.

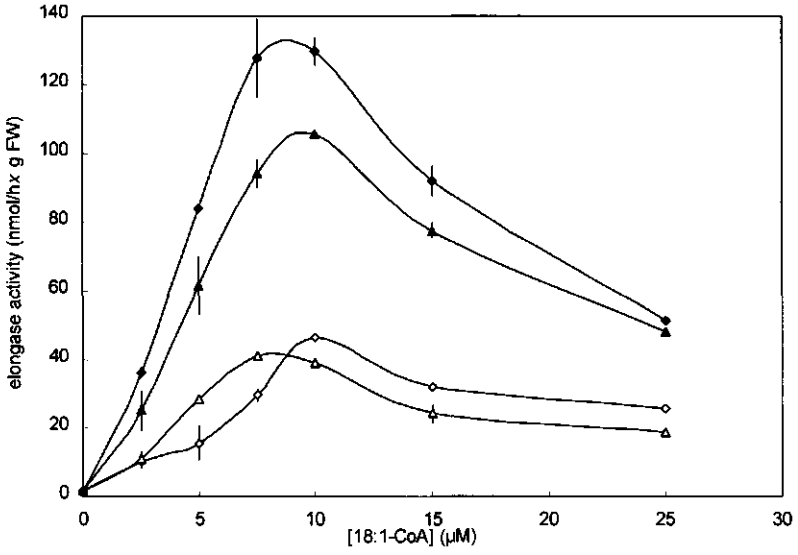


Fig 4.1: Effect of 18:1-CoA concentration on elongase activity. Activity is calculated as incorporation of malonyl-CoA after incubation at 30°C.
Diamonds: 15 °C; triangles: 25 °C. Closed symbols: + ABA; open symbols: -ABA.

Between enzyme preparations from MDEs grown at 15 or 25 °C and in the absence or presence of ABA differences were observed in maximum elongase activity based on embryo fresh weight. A lower growth temperature and addition of ABA resulted in increased activity. Due to the substrate inhibition Michaelis-Menten kinetics could not be obtained and therefore it was not possible to calculate an accurate K_m value.

For all enzyme preparations from MDEs grown under 4 different growth conditions, enzyme activity increased with a Q_{10} of about three over the range of 15 to 30 °C (table 4.2). This indicates that for these preparations the optimum temperature is higher than 30 °C and that they do not show differences in temperature sensitivity in this range. The distribution of

radioactivity, determined on TLC, over 20:1 and 22:1 was not influenced by either growth conditions or assay temperature (data not shown).

Table 4.2: Temperature dependence of elongase activity[1] from MDEs grown at different temperatures and in the absence or presence of ABA. Values are means ± SD from exp. 1.

	Assay temperature		
	15 °C	25 °C	30 °C
25 °C, - ABA	3.8 ± 0.3	11.3 ± 3.3	20.8 ± 2.9
25 °C, + ABA	9.9 ± 1.4	32.3 ± 5.3	59.2 ± 15.7
15 °C, - ABA	4.9 ± 0.8	18.2 ± 2.9	29.4 ± 5.5
15 °C, + ABA	12.5 ± 1.0	36.5 ± 6.5	62.5 ± 15.8

1: Elongase activity in nmol/h·g FW, determined in the presence of 8 µM [2-^{14}C]-malonyl-CoA and 25 µM 18:1-CoA.

Correlation between elongase activity and 22:1 accumulation.

In the previous section we have shown that the intrinsic enzyme characteristics of the different enzyme preparations are very similar, but that total activity based on embryo fresh weight is different. To determine the relationship between elongase activity and 22:1 content in the oil we compared it with 22:1 levels as a fraction of total fatty acids (% of FAs, table 4.3) and as absolute amounts (µmol/100 embryos, table 4.3 & fig 4.2).

Table 4.3: Correlation between elongase activity and the level of 22:1 produced in MDEs grown at different temperatures and in the absence or presence of ABA. Values are means ± SD from experiment 1. Relative values in brackets ("15 °C, + ABA" = 100).

	Elongase activity[1]	22:1 (µmol/100 emb)	22:1 (% of FAs)
25 °C, - ABA	39.2 ± 0.4 (30)	5.6 ± 0.5 (30)	17.1 ± 0.8 (51)
25 °C, + ABA	105.8 ± 0.2 (81)	13.4 ± 1.2 (72)	26.1 ± 0.9 (78)
15 °C, - ABA	46.6 ± 0.9 (36)	6.1 ± 1.4 (33)	23.6 ± 0.8 (71)
15 °C, + ABA	129.8 ± 4.1 (100)	18.8 ± 0.9 (100)	33.5 ± 0.7 (100)

1: Elongase activity in nmol/h·g FW, determined in the presence of 8 µM [2-^{14}C]-malonyl-CoA and 10 µM 18:1-CoA.

To facilitate comparison, data are normalised for the treatment showing the highest level of 22:1 or elongase activity. Elongase activities show only very

limited correlation with the level of 22:1 in % of FAs (R^2 = 0.44), but the correlation with absolute amounts of 22:1 is very high (R^2 = 0.97, fig 5.2).

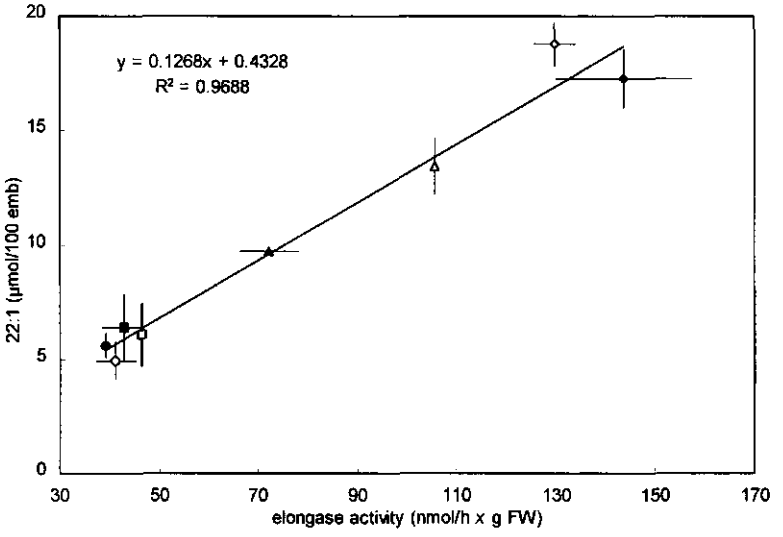


Fig 4.2: Correlation between elongase activity and the amount of 22:1 formed in mature seeds. Activity is calculated as incorporation of malonyl-CoA after incubation with 10 µM 18:1-CoA at 30°C.
Open symbols: experiment 1; closed symbols: experiment 2. Diamonds: 15 °C, + ABA; triangles: 25 °C, + ABA; squares: 15 °C, - ABA; circles: 25 °C, -ABA.

Acyltransferase activity

The effects of growth conditions on total acyltransferase activity were measured as incorporation of [U-^{14}C]-glycerol-3-phosphate, but only small differences were found (table 5.4). Phosphatidic acid (PA), diacylglycerol (DAG) and triacylglycerol (TAG) were identified on TLC in the product profile. Lysophosphatidic acid (LPA) could not be quantified separately as it migrates very close to phosphatidyl choline (PC, fig. 5.3) and both compounds together accounted for less than 5% of incorporated radioactivity. The main product observed was PA (60 to 70% of total radioactivity), with smaller amounts of DAG and TAG (table 5.4, fig 5.3).

Table 4.4: Correlation between total oil accumulated and acyltransferase activity in MDEs grown at different temperatures and in the absence or presence of ABA. "% TAG" and "% DAG + TAG" represent the fractions of total radioactivity incorporated in respective compounds. Values are means ± SD from experiment 1. Relative values in brackets ("15 °C, + ABA" = 100).

	Acyltransferase [1]	% TAG	% DAG + TAG	Oil[2]
25 °C, - ABA	147.7 ± 4.3 (85)	4.4 (100)	22.0 (73)	32.8 ± 0.8 (59)
25 °C, + ABA	186.4 ± 16.3 (108)	5.8 (132)	35.0 (116)	51.6 ± 5.6 (92)
15 °C, - ABA	170.5 ± 0.1 (99)	6.3 (143)	29.2 (97)	25.9 ± 5.1 (46)
15 °C, + ABA	173.0 ± 0.9 (100)	4.4 (100)	30.1 (100)	56.0 ± 2.1 (100)

1: Acyltransferase activity in nmol/h·g FW, determined in the presence of 6.5 µM [U-^{14}C]-G-3-P and 10 µM 18:1-CoA.

2: total oil expressed as µmol total fatty acids/100 emb.

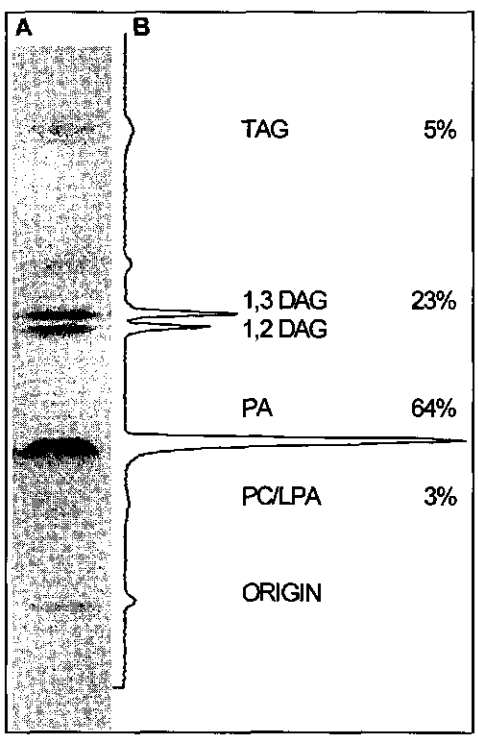


Fig 4.3: TLC analysis of acyltransferase products.
25 µg of protein from a 1000 *x* g fraction derived from MDEs grown at 15 °C, + ABA was incubated for 10 min with 6.5 µM [U-^{14}C]-G-3-P and 10 µM 18:1-CoA. Panel A: phosporimager picture of TLC lane, panel B: densitometric trace of radioactivity incorporated in reaction products.

Substrate inhibition of elongase activity: detergent effect of 18:1-CoA

Based upon characteristics of the response of elongase activity to 18:1-CoA concentration we analysed the detergent effect of 18:1-CoA. The substrate 18:1-CoA was able to solubilise some of the protein, which was detected as an increase in the fraction protein in the supernatant after centrifugation, and as a decrease in OD_{400} of about 10% (table 4.5), indicating a breakdown of membrane vesicles into smaller micelles (de Kruijff *et al.*, 1974).

Table 4.5: Effects of adding 50 µM 18:1-CoA on protein solubilisation and vesicle disruption. Protein solubilisation was determined as the percentage of protein in the supernatant after centrifugation (25 min, 15000 x g) of 100 µl HEPES buffer containing 25 µg protein and 150 µM Triton. Vesicle breakdown was determined as reduction of turbidity (OD_{400}) after addition of acyl-CoA to 50 µg protein in 1 ml HEPES buffer. Values are means ± SD.

	18:1-CoA absent	50 µM 18:1-CoA
Percentage protein in supernatant	9.7 ± 0.3	13.0 ± 0.9
Turbidity of suspension	0.62 ± 0.01	0.54 ± 0.02

When oleoyl-CoA was (partially) replaced with petroselinoyl-CoA (18:1 Δ6) in the elongase reaction, the latter was observed to be a substrate for elongase activity but with a lower conversion rate. Both fatty acyl-CoA esters were equally active as inhibitor for elongase activity (data not shown).

4.5 Discussion

Growth temperature and ABA have been shown to affect the level of 22:1 in the oil of MDEs as well as the level of elongase activity which catalyses the biosynthesis of this fatty acid. We observed that elongase reactions in the presence of 18:1-CoA showed maximum activity in the absence of ATP. Addition of ATP not only resulted in a reduction of total activity, but also in a shift in the ratio of the two products, 22:1 and 20:1. This effect is in contrast to observations by Hlousek-Radjocic *et al.* (1995), who concluded that an acyl-CoA ester was probably not the immediate substrate and that elongase activity is ATP-dependent. Our results are in accordance with those obtained in *Lunaria*

annua (Fehling & Mukherjee, 1991) and suggest that no energy dependent reaction is involved in the transfer of the acyl-chain from Coenzyme A to another carrier.

Another effect we observed is the reduction of elongase activity at higher concentrations of oleoyl-CoA. This may partly explain the observed effects of ATP. Addition of ATP might result in the formation of acyl-CoA from free fatty acids present in the membrane and small amounts of available free CoA, which could increase the concentration of acyl-CoA in the membrane above a critical level. The basis of the observed substrate inhibition of elongase activity might be a detergent effect of acyl-CoA; addition of oleoyl-CoA results in solubilisation of protein and a decrease in the amount of membrane vesicles (table 4.5). Similar inhibitory effects of petroselinoyl-CoA, which is a less efficient substrate for the elongase reaction, support the idea that the reduction in activity is probably due to disturbance of membrane integrity and not to inhibition at the active site of the enzyme.

Purification of the elongase complex, preferably to homogeneity, is not feasible as the enzyme rapidly loses its activity on solubilisation from the membrane (Fehling *et al.*, 1992). Therefore kinetic properties are approximations at best. In the preparations used it is impossible to determine whether increased activity is due to a more active enzyme (increased V_{max}) or to an increase in the amount of enzyme. However, the data allow us to get an indication of the way the elongase complex is regulated in response to both temperature and ABA.

The enzyme preparations derived from MDEs grown in the presence of ABA had higher activity than those derived from MDEs grown without ABA both when 18:1-CoA was varied (fig 4.1) and when assay temperature was varied (table 4.2). Also the enzyme preparations from MDEs grown at lower temperature had higher activity than those from MDEs grown at high temperature. Because of substrate inhibition by 18:1-CoA it was not possible to accurately determine K_m values. Therefore, we could not establish with

certainty whether these changes are due to an effect on the affinity of the elongase complex for oleoyl-CoA. However, the shape of the activation curve (fig 4.1) was the same for all preparations, suggesting that the affinity of the enzyme complex for its long-chain substrate is not influenced. According to available data control by malonyl-CoA seems unlikely, given the very broad range of malonyl-CoA concentrations that give high activity (Creach *et al.*, 1993). Also the similar reactions to changes in assay temperature (table 4.2) further indicate that characteristics of the enzyme complexes do not change. Thus our data suggest that the intrinsic characteristics of the elongase activity are not different in MDEs grown under the different culture conditions.

Regulation might occur at the level of total activity of the enzyme. A strong indication for such a regulation can be found in the high R^2 value observed in the correlation between elongase activity and the absolute level of 22:1 (fig 4.2). The relationship between elongase activity and the fraction of 22:1 in the oil is less clear (table 4.3), suggesting a role for another component, such as acyltransferase activity.

Effects of growth conditions on total acyltransferase activity were small in comparison to differences in total oil production (table 4.4) and these small effects on acyltransferase activity did not show high correlation with total oil accumulation. Therefore, the level of total acyltransferases does not appear to regulate the total amount of oil. Quantitation of the various acyltransferase products suggests that at this stage of development the activity of the PA-phosphatase is rate limiting, possibly sharing control with DAGAT, as the substrates of these two enzymes accumulate. Comparison of the accumulation of the products of these enzymes and oil production showed a similar lack of correlation as did total acyltransferase activity (table 4.4).

The above results strongly indicate that the effects of temperature and ABA on the amount of 22:1 are regulated directly at the level of the amount of elongase enzyme and not of intrinsic enzyme properties. Regulation of the fraction 22:1

in the oil probably also involves oil biosynthetic capacity. However, the mechanism of regulation of the total amount of oil in MDEs is still unclear, and does not appear to be dependent upon acyltransferase activity.

Acknowledgements

We like to thank Wilco Jordi and Eef-Jan Breukink for their suggestions in determining the possible mechanism for substrate inhibition by 18:1-CoA.

Chapter 5

ROLE OF THE RING METHYL GROUPS IN ABSCISIC ACID ACTIVITY IN MICROSPORE-DERIVED EMBRYOS OF OILSEED RAPE (Brassica napus L.)

Jeroen A. Wilmer, Suzanne R. Abrams, Johannes P.F.G. Helsper and Linus H.W. van der Plas

Keywords

Brassica napus L.; abscisic acid analogs; elongase; erucic acid; growth inhibition; hormonal activity; microspore-derived embryos.

5.1 Summary

In earlier investigations modification of the structure of abscisic acid (ABA) has been shown to result in a modification of the physiological activity. In this study we tested the effect of removal of three methyl groups on the ring of ABA and chirality on activity in microspore-derived embryos of oilseed rape (*Brassica napus* L.). The natural (+)-ABA molecule induces growth inhibition and an increase in the amount of erucic acid accumulated in the oil at medium concentrations less than 1µM. (-)-ABA shows similar activity. Removal of the 7'-methyl group results in a dramatic decrease in activity. (+)-7'-demethyl ABA retains some activity; 10 to 100 µM of this compound is needed for a response. (-)-7'-demethyl ABA is almost completely inactive. Similar effects are observed with regard to elongase activity, which catalyses erucic acid biosynthesis from oleic acid. Removal of the 8'- and 9'-methyl groups results in a more complex response. These compounds all show intermediate activity; for growth inhibition, the presence of the 9'-methyl is the more important determinant, whereas chirality dominates the response on erucic acid accumulation, with the (+)-enantiomer being the more stimulatory.

5.2 Introduction

In recent years a number of reports have been published on the activity of abscisic acid (ABA) derivatives in different plant systems. Activity was determined in combination with ABA, in some cases resulting in competitive inhibition (Wilen *et al.*, 1993), or separately to determine the relative activity in comparison with natural ABA. A number of different species and responses have been used to monitor ABA activity, like stomatal response and

transpiration (Jung & Grossmann, 1985, Blake *et al.*, 1990), chilling tolerance (Dorffling *et al.*, 1989, Churchill *et al.*, 1992), dormancy and germination (Walker-Simmons *et al.*, 1992 & 1995, Kim *et al.*, 1995), developmental processes (Abrams & Milborrow, 1991, Suttle & Abrams, 1993) and gene expression (Dong *et al.*, 1994, Hays *et al.*, 1996). Effects are expressed in relation to the activity of natural ABA in all these studies, as the sensitivity to ABA varies with the system and the response studied. Also the sensitivity to changes in the ABA structure depends upon the experimental system used.

It has previously been shown (Holbrook *et al.*, 1992, chapter 3) that addition of ABA can increase the amount of erucic acid (22:1) in oil of microspore-derived embryos (MDEs) of *Brassica napus* by about 10 mol%. In addition the total amount of oil increases. In this study we have compared the effects of a number of optically pure (+)- and (-)-enantiomers of ABA analogs (fig. 1), lacking one or two methyl groups from the ring (carbons 7', 8' or 9'), on oil accumulation and very-long chain fatty acid production in MDEs. We compared our data with those obtained by Walker-Simmons *et al.* (1995), who tested the effects of the same demethyl-analogs on wheat germination, to extend the knowledge on structural requirements of the ABA molecule for its interaction with receptors and physiological activity.

5.3 Materials & methods

Culture of microspore-derived embryos.

Plants of oilseed rape cultivar Reston, obtained from the University of Manitoba, were grown in the greenhouse at 20/17 °C (day/night) at a photo period extended to 16 hours with SON-T AGRO (Philips) to ensure induction of flowering. MDE culture was performed according to chapter 2. Briefly, racemes were collected at the onset of flowering and buds of 3.4 to 3.6 mm length were selected. The buds were sterilised in 2% NaClO and homogenised in a modified Lichter medium (NLN13). After filtration microspores were collected by

centrifugation, washed, diluted to 40000 spores ml^{-1} and 1 ml portions of suspension were plated in 35 mm Petri dishes. Embryogenesis was induced at 32 °C during three days, then cultures were transferred to 25 °C. Treatments were started at 350 °C·day (14 days in culture). Upon reaching a temperature sum of 450 °C·day embryos were rinsed with tap water and stored at -80 °C until analysis for elongase activity. Cultures were allowed to grow until 800 °C·day (31 days in culture) for the analysis of fatty acids.

ABA and analogs.

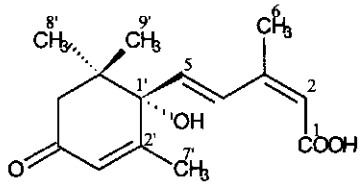
Optically pure analogs were synthesised and purified as described elsewhere (Walker-Simmons *et al.*, 1994, Rose *et al.*, 1996). Compounds were at least 99% pure. In this study we used 8 compounds (fig 1): both (+)- and (-)-enantiomers of natural ABA, of 7'-demethyl-ABA (7'-dm-ABA), of 8'-dm-ABA and of 8',9'-ddm-ABA. Treatments were started by replating the embryos in fresh medium containing ABA or its analogs, freshly diluted from 0.1 M stock solutions in methanol, controls were replated in medium containing 0.1‰ methanol.

Enzyme preparation and elongase assay.

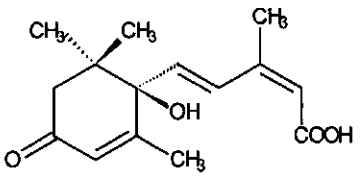
MDEs were ground in a buffer containing 80 mM HEPES, pH 6.8, 0.32 M sucrose, 10 mM ß-mercaptoethanol and 50 mg/ml PVPP at 5 ml of buffer per gram tissue. The homogenate was passed through 2 layers of miracloth and centrifuged at 1000 x g for 5 min. The supernatant was recentrifuged at 15000 x g for 25 min. The pellet was suspended in 80 mM Hepes, pH 6.8, 10 mM ß-mercapto-ethanol and centrifuged again for 25 min at 15000 x g.

Thirty µg of protein from the resuspended 15000 x g pellet was incubated at 30 °C in a buffer containing 80 mM HEPES, pH 7.2, 0.5 mM NADH, 0.5 mM NADPH, 2 mM DTT, 1 mM $MgCl_2$, 150 µM Triton X-100, 17 µM [2-^{14}C] malonyl-CoA (50 nCi) and 25 µM oleoyl-CoA in a final volume of 100 µl. After

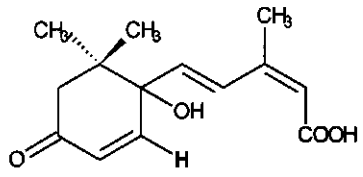
incubation for up to 30 min the reaction was stopped by addition of 100 µl 5N KOH, 10% methanol, and reaction products were saponified for 1.5 h at 70°C. Next 100 µl of 5M H_2SO_4, 10% malonic acid was added and the mixture extracted with 2 ml of chloroform. The organic phase was washed 3 times with 2 ml of water, evaporated under a stream of air and radioactivity was counted.



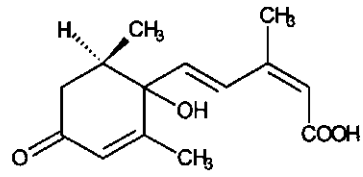
(S)-(+)-ABA



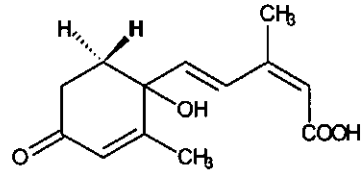
(R)-(-)-ABA



(+/-)-7'-demethyl-ABA



(+/-)-8'-demethyl-ABA



(+/-)-8',9'-didemethyl-ABA

Fig. 1: Chemical structure of ABA and demethyl ABA analogs. (+)- and (-)-enantiomers of demethyl analogs have the same chirality at C1' as the corresponding form of the intact molecule.

Lipid extraction and analysis of fatty acid composition

Oil was extracted from mature MDEs and fatty acid composition determined as fatty acid methyl esters as described previously (chapter 2). Briefly, embryos were homogenised in 5 ml of a mixture of methanol : chloroform : 0.01 N hydrochloric acid (2:1:0.8 by vol.), containing trimargarin as an internal standard. After shaking, chloroform and 0.01 N hydrochloric acid were added to obtain a mixture with a ratio 2:2:1.8 by volume. The suspension was centrifuged to achieve phase separation. The aqueous phase was washed once with 1.5 ml chloroform. Aliquots of organic fractions were applied to TLC plates which were developed in hexane : diethyl ether : acetic acid (70:30:1, by vol.), and lipids were visualised with iodine vapour. Areas containing triglycerides were scraped off and the powder transferred to tubes. Three ml 5% sulphuric acid in methanol was added, tubes were capped and incubated at 70 °C for three hours. Subsequently, fatty acid methyl esters (FAMEs) were extracted into hexane.

FAMEs were analysed using a capillary Chrompack CP9000 gas-chromatograph equipped with a CPwax 52 CB column and flame ionisation detection. The oven temperature was programmed from 190 °C (4.1 min) to 230 °C (4.4 min) at 20 °C min^{-1}. FAMEs were identified on the basis of retention time and quantified by electronic integration with reference to methyl margarate, derived from the internal standard trimargarin.

Chemicals.

All solvents used were HPLC or reagent grade. Radioactive malonyl-CoA was purchased from Amersham. ABA, buffers, cofactors and FAME-standards were purchased from Sigma and TLC-plates from Merck.

Calculation of $[PGR]_{50}$ values.

Dose-response curves were constructed for the different ABA analogs over the range of 0.01 to 100 µM, with the exception of (+)-ABA, where a range of 1nM

to 10 µM was used. $[PGR]_{50}$ values were calculated from sigmoidal data fits on these dose response curves, reflecting the transitional shape of the response. Because of variation between experiments and the limited resolution of the concentration range $[PGR]_{50}$ values are given in range classes.

5.4 Results

Effects of demethyl-analogs on embryo growth

Table 1: Relative growth inhibition and 22:1 increase by ABA derivatives and classification of concentrations resulting in half maximum reduction in fresh weight or half maximum increase in 22:1 ($[PGR]_{50}$). Values are means ± SD from three experiments.

	Growth inhibition		Increase in fraction 22:1	
	% at 10 µM[1]	$[PGR]_{50}$	% at 10 µM[2]	$[PGR]_{50}$
(+)-ABA	63 ± 15	<1 µM	29 ± 10	<1 µM
(-)- ABA	47 ± 27	<1 µM	28 ± 6	<1 µM
(+)-7'-dm-ABA	-1 ± 44	1-10 µM	14 ± 5	10-100 µM
(-)-7'-dm-ABA	-21 ± 21	10-100 µM	-1 ± 8	> 100 µM
(+)-8'-dm-ABA	27 ± 27	1-10 µM	22 ± 13	1-10 µM
(-)-8'-dm-ABA	24 ± 25	1-10 µM	11 ± 5	10-100 µM
(+)-8',9'-ddm-ABA	41 ± 20	<1 µM	26 ± 11	1-10 µM
(-)-8',9'-ddm-ABA	49 ± 20	<1 µM	11 ± 13	10-100 µM

1: Control values: 356-935 mg/100 emb., 45-60% growth inhibition at saturating concentrations.
2: Control values: 19.4-31 mol% 22:1, max. 22:1 increase 25-30% at saturated response.

The capacity of demethyl-analogs of ABA to inhibit growth of MDEs is shown in table 1. At 10 µM the (+)- and (-)-enantiomers of ABA and their 8',9'-didemethyl analogs were equally active in inhibiting embryo growth. Both 8'-demethyl enantiomers showed reduced inhibitory activity (about 50%) and the (-)-7'-demethyl analog was even less active. The extremely large SD on the value for (+)-7'-demethyl-ABA indicates that the threshold concentration for activity of this compound is about 10 µM. The impact of the 7'-methyl group is also illustrated in the $[PGR]_{50}$ values for these compounds which show that, in comparison with most other analogs, high concentrations of the 7'-demethyl analogs are required for half-maximum activity (table 1).

Accumulation of erucic acid

Accumulation of erucic acid can be calculated in two ways, as a fraction of the oil (% of total fatty acids) or as absolute amount accumulated (µmol 22:1/ 100 embryos). To induce effects on oil composition high concentrations of some analogs are required which result in a strong reduction in embryo growth (see above). When effects of ABA analogs are calculated as changes in the absolute amount of 22:1, increases induced by the addition of compound tested are at least partially masked by a reduction in growth, as illustrated for (+)-8'-demethyl-ABA (fig 2). The (-)-7'-demethyl analog showed no effect while the other analogs all appeared to have at least some ABA activity, with (+)-ABA as the most active compound (data not shown).

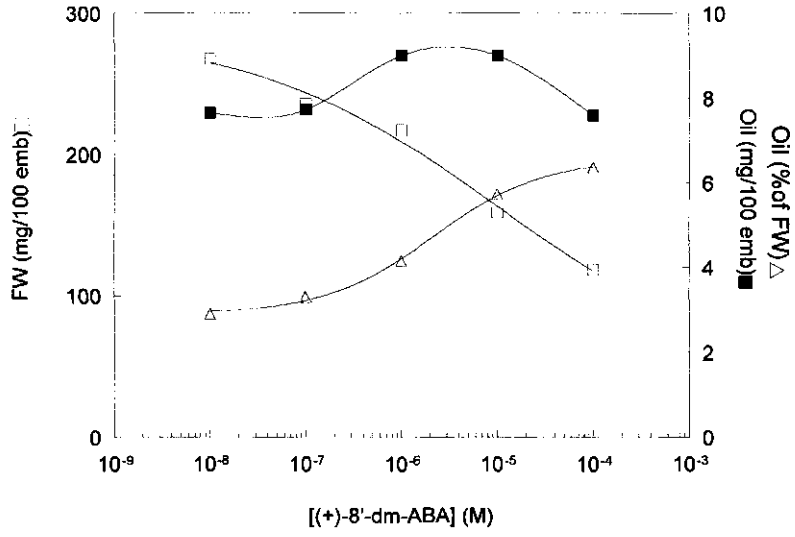


Fig. 2: Effect of (+)-8'-dm-ABA concentration in the medium on fresh weight (□, left axis), absolute amount of oil accumulated (■, right axis) and on oil accumulation as % of FW (△, right axis) in microspore-derived embryos of *Brassica napus* L. cv Reston.

Changes in the fraction 22:1 in the oil due to application of ABA analogs are more easily interpreted (table 1). Again both enantiomers of intact ABA have

reached maximum activity, at 10 µM, as is also observed for (+)-8',9'-ddm ABA and (+)-8'-ABA. The (-) enantiomers of these analogs show intermediate activity as does (+)-7'-dm-ABA. The (-)-7'-dm analog is completely inactive. The $[PGR]_{50}$ values with respect to 22:1 accumulation show a similar order of activity, although (+)-8'-dm- and (+)-8'9'-ddm-ABA are somewhat less active than (+)- and (-)-ABA (table 1).

Elongase activity.

Table 2: Elongase activity at 450 °C·day and amount of 22:1 at 800 °C·day after treatment of MDEs with 10 µM of each ABA analog. Data are means ± SD from the same experiment.

	Elongase activity (nmol/h·g FW)	Amount of 22:1 (µmol/100 emb)
Control	26.3 ± 1.9	70.8 ± 11.6
(+)-ABA	61.6 ± 0.9	124.2 ± 22.5
(-)- ABA	77.8 ± 11.3	137.9 ± 5.4
(+)-7'-dm-ABA	50.7 ± 9.7	80.2 ± 8.2
(-)-7'-dm-ABA	24.9 ± 0.8	59.0 ± 12.3
(+)-8'-dm-ABA	90.3 ± 6.5	114.3 ± 21.3
(-)-8'-dm-ABA	32.7 ± 0.2	132.7 ± 21.3
(+)-8',9'-ddm-ABA	40.8 ± 4.8	190.8 ± 35.5
(-)-8',9'-ddm-ABA	63.5 ± 10.5	166.1 ± 0.5

Because of the large differences between batches of MDEs and limited batch size we only determined elongase activity at 10 µM for each analog. The maximum effect induced by (+)-8'-dm-ABA resulted in a 3½-fold increase in elongase activity, whereas addition of (+)-ABA only doubled elongase activity (table 2). The effects of (+)-ABA, (-)-ABA and (-)-8',9'-ddm-ABA were similar and (-)-7'-dm-ABA was completely inactive in this assay. The remaining compounds showed intermediate activity resulting in about 50% increase in activity.

Correlation of elongase activity and 22:1 accumulation

No significant correlation (R^2 ~ 0.1) was observed between elongase activity and the amount of 22:1 accumulated for the various ABA-analogs. When only the data obtained with (+)- and (-)-ABA enantiomers and their 7'-demethyl analogs are considered, together with the control, the correlation is much higher (R^2 ~ 0.9). A similar effect is also observed for the correlation of elongase activity with the fraction 22:1 in oil (R^2 ~ 0.3 and ~ 0.8 respectively).

5.5 Discussion

Our results indicate that the role of the 3 ring-methyl groups in ABA activity on growth and 22:1 accumulation in MDEs of oilseed rape is similar to the effects described for wheat germination (Walker-Simmons *et al.*, 1994).

Removal of the 7'-methyl group results in a reduction in ABA activity in both systems. Only at high concentrations, with a $[PGR]_{50}$ of 10 µM or higher, (+)-7'-dm-ABA, with the same chirality as natural ABA, has limited activity both in inhibiting growth and in increasing 22:1. However, the response at 100 µM is similar to that obtained with (+)-ABA. The compound with the opposite chirality, (-)-7'-dm-ABA, only showed an effect on growth at the highest concentration and has no effect on 22:1 accumulation. In wheat germination studies both compounds are completely inactive (Walker-Simmons *et al.*, 1994). Both enantiomers had effects on elongase activity similar to those on 22:1 accumulation; (+)-7'-demethyl-ABA induced a small increase in activity and its (-)-isomer was completely inactive. If we combine these data and determine correlations as was done for elongase activity and absolute amount 22:1 in the oil, consistent effects are observed. This suggests that either one ABA receptor is involved in transmitting hormonal signals for effects on growth and oil composition in MDEs of oilseed rape or, if more than one receptor is involved, all receptors require a methyl-group in the 7'-position for recognition.

All 8'- and 8',9'-demethyl analogs of ABA show activity both with regard to growth inhibition and erucic acid level (table 1). For growth inhibition the effects are similar to those found by Walker-Simmons *et al.* (1994) using the wheat cv Brevor: the 8',9'-didemethyl analogs are somewhat more active than the 8'-demethyl analogs, but no differences can be found between chiral forms. The effects on the level of 22:1 suggest a clear effect of chirality, but little effect of removing the second methyl-group: the effects at 10 µM are identical and $[PGR]_{50}$ values fall in the same range. Elongase activity data are more difficult to interpret: (-)-8'9'-didemethyl-ABA is more active in inducing elongase activity than its (+)-isomer, while (+)-8'-demethyl-ABA is more effective than its (-)-enantiomer, in fact it is the most active compound in inducing elongase, but causes only intermediate increases in the absolute level of 22:1. This lack of relation between elongase activity and 22:1 accumulation suggests that the effects on elongase activity can not be properly assessed at 450 °C·day, as far as these analogs are concerned (see chapter 4). Whether this is due to an interaction between induction of oil accumulation and inhibition of growth as demonstrated in fig. 2, or to a shift in response timing caused by the removal of the 8'- and 9'-methyl group in the ABA analogs, can not be concluded from the present data. The fact that the effects on growth and on 22:1 accumulation are different suggests the presence of at least 2 receptors for ABA in MDEs: one involved in regulation (or termination) of growth of the embryo, and the other involved in regulation of oil composition and accumulation. The combination of the separate signal-transduction pathways originating from these two receptors then results in the observed differential effects.

Chapter 6

GENERAL DISCUSSION

6.1 Metabolic costs and adaptations required for erucic acid accumulation

This thesis describes the effects of temperature and ABA on oil composition and enzyme activities involved in oilseed rape. Especially the data presented in chapter 4 challenge the development of a regulation model explaining the observed effects. In this chapter such a model will be presented after a discussion of the possible physiological roles of erucic acid.

Production of very long chain fatty acids (VLCFAs) not only requires extra energy, but also adaptations in the enzymes to synthesise and incorporate these compounds. In case of 22:1 and 20:1, enzymes must be produced that elongate oleic acid formed in the plastid, a source of malonyl-CoA must be available and the enzymes involved in glycerolipid synthesis must be able to discriminate between "normal" long chain fatty acids, incorporated in both membrane phospholipids and storage TAG, and the specific VLCFAs observed only in seed oil.

In *Brassica* elongation of 18:1 occurs in the ER on acyl-CoA esters (Fehling & Mukherjee, 1991). This reaction is very similar to the production of VLCFAs and fatty alcohols for wax production (Post-Beittenmiller, 1996). The homology between these two systems suggests that the enzymes used for wax synthesis have been modified in expression pattern and substrate specificity to perform this function. Since production of erucic acid does not occur in vegetative tissue a separate set of enzymes must have evolved, that is only expressed in seeds.

Another adaptation of lipid metabolism is the production of acyltransferases and head-group introducing enzymes that are able to discriminate between different types of fatty acids, since erucic acid has adverse effects on membrane characteristics and will therefore be excluded from membranes. In most *Brassica* species the LPAAT completely excludes VLCFAs, although lines of *B. oleracea* have been found to incorporate 22:1 in the sn-2 position to a limited extent (Taylor *et al.*, 1995). Phosphatidic acid and diacylglycerol can thus contain VLCFAs in the sn-1 position, and a mechanism must exist to exclude

these moieties from membrane lipids in favour of moieties containing normal long chain fatty acids at both sn-1 and sn-2.

One way to exclude undesirable fatty acids from the membranes has been described for transgenic oilseed rape containing lauric acid (12:0; Eccleston *et al.*, 1996). In this report it was shown that excess 12:0 was removed from the system by breakdown of fatty acids and lipids containing 12:0. This route results in net losses of energy, and therefore in a loss of relative fitness. Whether a similar pathway is used to remove excess 22:1 is not clear. Even if no extra fatty acids are produced and then broken down in a futile cycle to maintain membrane characteristics, the production of VLCFAs comprises an investment in additional and more specialised enzymes.

6.2 A possible role for erucic acid in oilseed rape

In the studies presented in chapters 2 and 3 it was shown that the 22:1 level can vary considerably in the cultivar Reston, depending upon temperature and ABA level. In Gulle the level of 22:1 is much less sensitive to external conditions. The levels of eicosenoic acid (20:1) remain relatively stable for both Reston and Gulle. So far the issue of a functional aspect for these changes has not been addressed, but first a role for 22:1 has to be established. Feasible functions of 22:1 accumulation in the oil may include: improvement of physical properties of the oil, enhanced energy supply upon mobilisation and protection against herbivores or pathogens. Aspects of these functions will be discussed below.

The effects of growth temperature on 22:1 content suggest some effect on the fluidity of the oil. To obtain a stable fluid oil, a higher proportion of shorter chains or more highly desaturated fatty acids is required at a lower temperature. If the balance between 18:1 and 22:1 is part of such a mechanism, the level of 22:1 would be reduced at a lower temperature. The data presented in chapters 2 and 3 clearly show the opposite. Another factor

making a role in fluidity control through fatty acid composition improbable is the fact that fatty acids stored in oil cannot be remobilised and modified once they are incorporated in triacylglycerols and stored in oil bodies, which means that control can only be exerted at the time of synthesis. Also this phenomenon was addressed in chapter 2.

A second possible role for 22:1 might be found in enhancing the energy efficiency per unit of oil. This can be seen as either rate of mobilisation of fatty acids or as amount of energy released per gram of oil. Comparison of the fatty acid composition of Aurora and Reston seeds grown at 15 °C (chapter 2, table 1) shows a difference in 22:1 level of 40%, resulting in average formula weights for TAG of 879 Da for Aurora and 955 Da for Reston. When we calculate the number of TAG molecules per gram oil from these figures, we find 8.6% less TAG molecules per gram in Reston. This means that in seeds of Reston 8.6% less molecules have to be hydrolysed for a similar amount of oil, resulting in either a faster mobilisation of fatty acids or a reduced requirement for lipase enzyme. To my knowledge no comparison has ever been made between near-isogenic lines containing different levels of 22:1 on germination behaviour. However, one study shows that more erucic acid in the oil correlates with later and not earlier stem elongation (Sleiman *et al.*, 1987). The underlying mechanism is unclear, but these results appear to contradict possible effects of erucic acid level on enhanced mobilisation described above.

When calculating the amount of energy stored in the fatty acids, the differences are small. Assuming complete breakdown by the oxidative pathway it is possible to calculate ATP yield (Stryer, 1981: chapter 17). Using this approach, degradation of a typical TAG molecule of Reston produces 11.4% more ATP than that of Aurora, assuming that the glycerol does not contribute. When taking into account the lower number of TAG molecules per gram oil in Reston, the difference in energy produced is reduced to about 2.6%. This small difference in energy released with a large difference in VLCFAs (50% in Reston

and 1% in Aurora), indicates that the extra amount of energy released is negligible.

It has been shown that erucic acid has detrimental effects on health and development of a number of animal species (Kramer *et al.*, 1990), although effects on humans are less clear (Laryea *et al.*, 1992). Especially cardiac muscle and vascular tissue are affected, resulting in a slow reduction of fitness. A role for 22:1 as antifeedant would require a much faster effect on the browsing animal (*e.g.* like ricinoleic acid from castor oil), as only an immediate effect will result in avoidance of the plant. In this context glucosinolates, also found in oilseed rape, are much more effective. This suggests that 22:1 is not a primary antifeedant against herbivores in oilseed rape.

On the other hand, levels of 22:1 correlate positively with resistance to a number of pathogens like downy mildew (Nashaat & Rawlinson, 1994) and *Phoma* (Kruger, 1982). A possible mechanism explaining both high erucic acid and pathogen resistance might be a general increase in elongase activity in the entire plant, giving rise to both VLCFA production in the seed and increased wax production on the leaves. The increased wax production then enhances resistance to pathogens (Anonymous, 1976). Based on the fact that it has been the only gene for a subunit of the elongase complex found by mutation so far and the possibilities to induce VLCFA synthesis by transformation with just this gene, Millar and Kunst (1997) suggested that only the ß-ketoacyl-synthase unit is specific to the different elongase complexes and that other units are shared between complexes.

Neither of these possible functions show a well defined role for erucic acid in *Brassica* seed. Its accumulation may be a neutral change, not affecting the fitness of the plant containing 22:1 in its oil. This poses the question why VLCFAs are still present in seeds of *Brassicaceae*.

6.3 A model to explain the mechanism controlling erucic acid levels

The effects described in chapters 2 and 3 not only included effects on the fraction of 22:1 in the oil, but also on the total amount of oil accumulated. Therefore a regulation model must not only include elongation, but also biosynthesis of fatty acids and incorporation in the oil. The effects of growth temperature in seeds and MDEs, described in chapter 2, were very similar with regard to changes in the fraction 22:1, but absolute levels of 22:1 were different as well as the effects on the total level of oil. In MDEs the effects on the total amount of oil were negligibly small, especially when compared to those in seeds. Together with smaller changes in the absolute amount of 22:1 in MDEs this resulted in a similar increase in the fraction of 22:1 with the lower temperature in both systems (about 10% upon transfer to 15°C). These observations suggest that the balance between oil accumulation and 22:1 synthesis is an important factor in regulation of oil quality. In chapter 3 it was shown that ABA also influences oil composition in MDEs, but in a different way. The level of oil as well as the fraction 22:1 increases upon ABA application, whereas growth temperature had little effect on total oil. Another observation made in these chapters is the increase in desaturation with lower temperature, a phenomenon described previously (Trémolieres *et al.*, 1982).

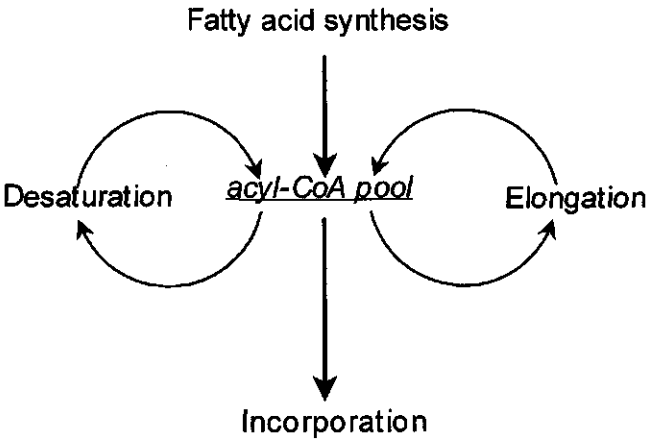


Figure 6.1: Central position of the acyl-CoA pool in lipid synthesis.

All these effects of environmental conditions on fatty acid composition influence the size, composition and turnover of the acyl-CoA pool (and the PC/DAG pool that is in equilibrium with it through acyl-exchange) used for the assembly of phospholipids for membranes and TAG as a storage compound. Thus the acyl-CoA pool becomes central in regulation of oil quality and quantity (fig 6.1).

Therefore, to get a better insight into the regulation of oil composition and oil quantity we have to study the enzymes determining the size and composition of the acyl-CoA pool in seeds and MDEs. The data on oil accumulation shown in chapter 2 (figs 2.2 & 2.4) together with the effects of temperature switch (fig 2.3) indicated that accumulation rates approach maximum levels between 400 and 600 °C·day and that temperature effects occur at the same time. Therefore the activity of the enzymes involved is expected to be maximal in the same timeframe and enzyme activities were measured in this timeframe to determine the mode of regulation.

A first point of control is at the level of carbon allocation: carbohydrates can be stored as starch or are converted into protein and lipids. Carbon allocation does not seem to be affected by growth temperature as the total amount of oil invariably comprises about 40% of dry matter both in seeds and MDEs (results not shown). The exact control point is unknown, but candidates are acetyl-CoA carboxylase (ACCase), the first committed step in fatty acid biosynthesis (Ohlrogge & Browse, 1995), and diacylglycerol acyltransferase (DAGAT), the last and possibly rate limiting enzyme in oil synthesis (Murphy, 1988). Data presented in chapter 4 (table 4.4) suggest that the latter or any of the other acyltransferases are not the key enzymes for control, at least in MDEs of 450 °C·day old, since the correlation between enzyme activity and total oil produced is low. Therefore the size and turnover of the acyl-CoA pool will probably be determined by the activity of the ACCase or one of the other components of the fatty acid synthase complex.

As both G-3-P acyltransferase and DAGAT have rather broad substrate specificities (Cao & Huang, 1987), regulation of fatty acid composition of the final product, TAG, by selective incorporation of fatty acids seems unlikely. If selectivity of acyltransferases does not determine the fatty acid composition of TAG, the composition of the acyl-CoA pool providing substrates is important. A number of enzymes together determines which fatty acids accumulate in this pool: desaturases, which catalyse the synthesis of 18:2 and 18:3 from 18:1, and elongase, which catalyses its elongation to 20:1 and 22:1. The mode of regulation of desaturases is still unclear, although different hypotheses exist (Trémolieres *et al.*, 1982, Browse & Slack, 1983), with at least one of them assuming a differential level of expression resulting in different levels of desaturation.

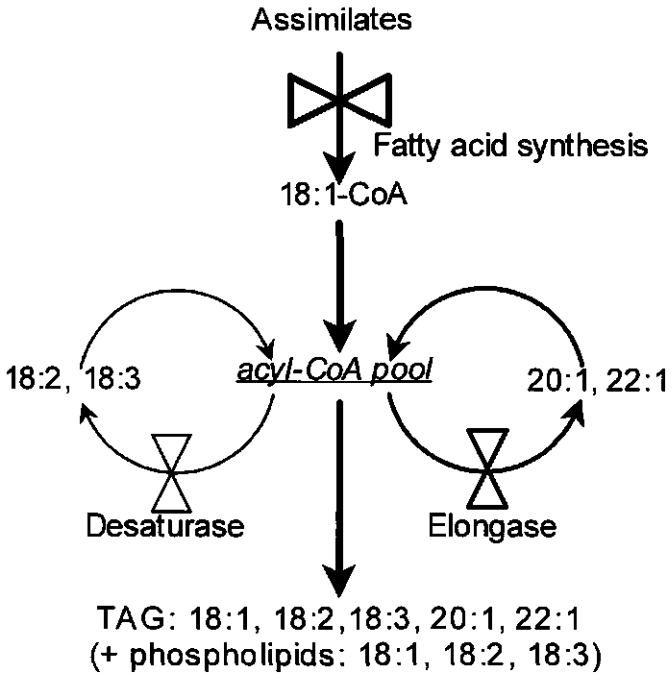


Figure 6.2: Oil composition is determined by the relative activity of the enzymes contributing to the acyl-CoA pool in lipid synthesis. (Thickness of arrows indicates flux through respective parts of metabolism in high erucic acid oilseed rape)

Data in chapter 4 suggest that the intrinsic characteristics of the elongase complex are not affected by temperature or ABA (fig 4.1, table 4.2), but that the total activity changes due to synthesis of enzyme and correlates closely with the amount of 22:1 formed (fig 4.2, table 4.3).

Thus, if the amount of 22:1 formed is not regulated by the affinity of the elongase complex for its substrate 18:1-CoA, but the amount of enzyme is limiting the synthesis of 22:1, the absolute amount of 18:1-CoA is not critical to the activity of the elongase complex. In the presence of a large excess of 18:1-CoA, most of this substrate will not be transformed into 22:1, whereas if the acyl-CoA pool is small a larger proportion will be converted into 22:1. Thus the size of the acyl-CoA pool becomes important in determining the fraction 22:1 produced in the oil: similar levels of elongase activity, and consequently of 22:1, can give rise to different fractions of 22:1 in the oil if the rate of oil synthesis is different. As already mentioned previously, the enzymes incorporating either 18:1 or 22:1 in TAG do not discriminate between these fatty acid species. Combination of the control of acyl-CoA pool size and the control of 22:1 production leads to a model which is based on flux control.

In this model the composition of the acyl-CoA pool is determined by the relative activities of enzymes contributing to and drawing from this pool (fig 6.2). An increase in the activity of one component leads to changes in overall fatty acid composition, because it influences the size or turnover of the entire acyl-CoA pool. The results obtained with 8'-demethyl- and 8',9'-didemethyl-analogs (chapter 5) further increase the complexity of regulation, emphasising the equal importance of total flux and elongase activity. These ABA analogs limit the total carbon flux at high concentrations, resulting in reduced growth and oil accumulation and only moderate increases in the absolute amount of 22:1 compared to control values. However, the fraction 22:1 remains high and relatively stable at these high concentrations of the analogs. At the same time

8',9'-didemethyl-ABA, less effective in inducing elongase, had similar effects on the fraction 22:1 as 8'-demethyl -ABA.

Studies with *Arabidopsis* support this model: reduction of DAGAT activity in a mutant resulted in a large increase in the fraction 18:3 in the oil, due to reduced oil synthesis (and elongation, supposedly by feedback inhibition from excess, free 20:1-CoA; Katavic *et al.*, 1995), resulting in high levels of 18:1-CoA and DAG and a slow turnover of these pools, enabling desaturases to process a larger fraction of the fatty acids produced.

6.4 Perspectives for regulation and modification

In the second section of this chapter it has been shown that even large differences in the amount of very long chain fatty acids in the oil only have minor effects on plant fitness. This suggests that the regulation of erucic acid levels in the range observed in the studies presented in this thesis does not confer any additional fitness to the plant and therefore appears to be a neutral modification, as is the presence of VLCFAs itself.

The close linkage between membrane quality and the level of polyunsaturated fatty acids in oil does not always allow large scale modification of the level of polyunsaturates, as it might have implications on plant fitness, as has been shown for sensitivity to both high and low temperatures in *Arabidopsis thaliana* (Hugly *et al.*, 1989, Miquel & Browse, 1994). Such adverse effects on membrane characteristics will be of little or no importance in attempts to increase the level of VLCFAs in seed oil above the current levels. The limit posed upon 22:1 accumulation in oilseed rape by the selectivity of the LPAAT has been broken by transformation with a LPAAT from a species that can incorporate this fatty acid in the sn-2 position (Lassner *et al.*, 1995). The next step will be an enhancement of elongase activity in the seeds, as this enzyme has been shown to be limiting in the accumulation of 22:1. Increasing the level of elongase activity will probably not result in oil containing only 22:1, as about

10 mol% of 20:1 has been found in all seeds and MDEs in this study and in a genetic study on the heredity of 22:1 level (Jönsson, 1977). Also some polyunsaturated fatty acids will remain present due to the fact that the desaturases producing them can not be eliminated as mentioned previously, and will therefore always use some of the 18:1 exported from the plastid.

Nevertheless, levels of up to 80 or 85% of erucic acid appear to be feasible. However, the effects on plant growth are unpredictable as both oil synthesis and synthesis of membrane lipids appear to use the same pool of acyl-CoA and Kennedy-pathway intermediates. The effects of a large proportion of VLCFAs in both sn-1 and sn-2 of key intermediates of membrane synthesis might include the induction of a futile cycle of VLCFA synthesis and breakdown as described for 12:0 (Eccleston *et al.*, 1996), but these types of effects will only appear once such high levels of 22:1 in intermediates occur. If these effects do not alter plant fitness to a large extent, 22:1 derived from seed oil can become a highly valuable replacement for fossil oil in plastic synthesis and (bio-) lubricants.

References

Abrams, S.R. and B.V. Milborrow: Synthesis and biological activity of allenic analogues of abscisic acid. Phytochemistry 30: 3189-3195 (1991).

Albrecht, S., C. Möllers and G. Röbbelen: Selection for fatty acid composition in microspore-derived embryoids (MDE) of rapeseed, *Brassica napus* (L.). J. Plant Physiol. 143, 526-529 (1994).

Anonymous: Rape. UK, Rothamsted Experimental Station: Report for 1975. Part 1. 371pp. Lawes Agricultural Trust, Harpenden, UK (1976).

Arondel, V., B. Lemieux, I. Hwang, S. Gibson, H.M. Goodman and C. Somerville: Map-Based Cloning of a Gene Controlling ω-3 Fatty Acid Desaturation in Arabidopsis. Science 258: 1353-1355 (1992).

Bafor, M., L. Jonsson, A.K. Stobart and S. Stymne: Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. Biochem. J. 273: 31-38 (1990)

Battey, J.F. & J.B. Ohlrogge: A comparison of the metabolic fate of fatty acids of different chain lengths in developing oilseeds. Plant Physiol. 90, 835-840 (1989).

Bernerth, R. and M. Frentzen: Utilization of erucoyl Coenzyme A by acyltransferases from developing seeds of *Brassica napus* (L.) involved in triacylglycerol biosynthesis. Plant Sci. 67: 21-28 (1990).

Blake, T.J., W. Tan and S.R. Abrams: Antitranspirant action of abscisic acid and ten synthetic analogs in black spruce. Physiol. Plant. 80: 365-370 (1990).

Bramley, P.M.: Carotenoid biosynthesis: a target site for bleaching herbicides. Biochem. Soc. Trans. 22: 625-629 (1994).

Brown, A.P., C.L. Brough, J.T.M. Kroon and A.R. Slabas: Identification of a cDNA that encodes a 1-acyl-sn-glycerol-3-phosphate acyltransferase from *Limnanthes douglasii*. Plant Mol. Biol. 29: 267-278 (1995).

Browse, J.A. and C.R. Slack: The effect of temperature and oxygen on the rates of fatty acid synthesis and oleate desaturation in safflower (*Carthamus tinctorius*) seed. Biochim. Biophys. Acta 753: 145-152 (1983).

Bruijn, S.M. de: Abscisic acid and assimilate partitioning during seed development. PhD-thesis, Wageningen Agricultural University (1993).

Cahoon, E.B. and J.B. Ohlrogge: Metabolic evidence for the involvement of a Δ-4-palmitoyl-acyl carrier protein desaturase in petroselinic acid synthesis in Coriander endosperm and transgenic Tobacco cells. Plant Physiol. 104: 827-837 (1994).

Cahoon, E.B., C.K. Becker, J. Shanklin and J.B. Ohlrogge: cDNAs for isoforms of the Δ-9-stearoyl-acyl carrier protein desaturase from *Thunbergia alata* endosperm. Plant Physiol. 106: 807-808 (1994).

Cahoon, E.B., J. Shanklin and J.B. Ohlrogge: Expression of a Coriander Desaturase Results in Petroselinic Acid Production in Transgenic Tobacco. Proc. Natl Acad. Sci. 89: 11184-11188 (1992).

Canvin, D.T.: The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. Can. J. Bot. 43, 63-69 (1965).

Cao, Y.Z. and A.H.C. Huang: Acyl coenzyme A preference of diacylglycerol acyltransferase from the maturing seeds of cuphea, maize, rapeseed and canola. Plant Physiol. 84: 762-765 (1987).

Chen, J.L. and W.D. Beversdorf: Evaluation of microspore-derived embryos as models for studying lipid biosynthesis in seed of rapeseed (*Brassica napus* L.). Euphytica 58, 145-155 (1991).

Churchill, G.C., B. Ewan, M.J.T. Reaney, S.R. Abrams and L.V. Gusta: Structure-activity relationships of abscisic acid analogs based on the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss) cell cultures. Plant Physiol. 100: 2024-2029 (1992).

Cordewener, J.H.G., R. Busink, J.A. Traas, J.B.M. Custers, H.J.M. Dons & M.M.V. Campagne: Induction of microspore embryogenesis in *Brassica napus* L. is accompanied by specific changes in protein synthesis. Planta 195, 50-56 (1994)

Cottingham, I.R., A. Austin, C. Sidebottom and A.R. Slabas: Purified enoyl-[acyl-carrier-protein] reductase from rape seed (*Brassica napus*) contains two closely related polypeptides which differ by a six-amino-acid N-terminal extension. Biochim. Biophys. Acta 954: 201-207 (1988).

Creach, A., R. Lessire and C. Cassagne: Kinetics of C18:1-CoA elongation and transacylation in rapeseeds. Plant Physiol. Biochem. 31: 923-930 (1993).

Custers, J.B.M., J.H.G. Cordewener, Y. Nollen, H.J.M. Dons & M.M.V. Campagne: Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep. 13, 267-271 (1994).

Davies, H.M., D.J. Hawkins and J.S. Nelsen: Lysophosphatidic acid acyltransferase from immature coconut endosperm having medium chain length substrate specificity. Phytochemistry 39: 989-996 (1995).

Dong, J.Z., G. Pilate, S.R. Abrams and D.I. Dunstan: Induction of a wheat Em promoter by ABA and optically pure ABA analogs in white spruce (*Picea glauca*) protoplasts. Physiol. Plant. 90: 513-521 (1994).

Dorffling, K., A. Flores, A. Grau and B. Capell: Effect of new synthetic abscisic acid analogues in combination with tetcyclacis on chilling and freezing resistance in crop plants. Acta Hortic. 239: 187-196 (1989).

Dormann, P., F. Spener and J.B. Ohlrogge: Characterization of 2 acyl-acyl carrier protein thioesterases from developing *Cuphea* seeds specific for medium-chain-acyl and oleoyl-acyl carrier protein. Planta 189: 425-432 (1993).

Downey, R.K. & B.M. Craig: Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus* L.). J. Am. Oil Chem. Soc. 41, 475-478 (1964).

Eccleston, V.S., A.M. Cranmer, T.A. Voelker and J.B. Ohlrogge: Medium-chain fatty acid biosynthesis and utilization in *Brassica napus* plants expressing lauroyl-acyl carrier protein thioesterase. Planta 198: 46-53 (1996).

Evenson, K.J. and D. Post-Beittenmiller: Fatty acid-elongating activity in rapidly expanding leek epidermis. Plant Physiol. 109: 707-716 (1995).

Fawcett, T., W.J. Simon, R. Swinhoe, J. Shanklin, I. Nishida, W.W. Christie and A.R. Slabas: Expression of mRNA and steady-state levels of protein isoforms of enoyl-ACP reductase from *Brassica napus*. Plant Mol. Biol. 26: 155-163 (1994).

Fehling, E., and K.D. Mukherjee: Acyl-Coenzyme A elongase from a higher plant (*Lunaria annua*): metabolic intermediates of very-long-chain Acyl-Coenzyme A products and substrate specificity. Biochim. Biophys. Acta 1082: 239-246 (1991).

Fehling, E., R. Lessire, C. Cassagne and K.D. Mukherjee: Solubilization and partial purification of constituents of acyl-CoA-elongase from *Lunaria annua*. Biochim. Biophys. Acta 1126: 88-94 (1992).

Finkelstein, R.R. and C.R. Somerville: Three classes of abscisic acid (ABA)-insensitive mutations of Arabidopsis define genes that control overlapping subsets of ABA responses. Plant Physiol. 94 1172-1179 (1990).

Finkelstein, R.R., and C. Somerville: Abscisic acid or high osmoticum promote accumulation of long-chain fatty acids in developing embryos of *Brassica napus*. Plant Sci. 61, 213-217(1989).

Finkelstein, R.R., K.M. Tenbarge, J.E. Shumway & M.L. Crouch: Role of ABA in maturation of rapeseed embryos. Plant Physiol. 78, 630-636 (1985).

Firn, R.D.: Growth substance sensitivity: the need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. 67: 267-272 (1986).

Fox, B.G., J. Shanklin, C. Somerville and E. Munck: Stearoyl-acyl carrier protein D9 desaturase from *Ricinus communis* is a diiron-oxo protein. Proc. Natl Acad. Sci. 90: 2486-2490 (1993).

Fuhrmann, J. & K.P, Heise: Factors controlling medium-chain fatty acid synthesis in plastids from maturing *Cuphea* embryos. Z. Naturforsch. 48, 616-622 (1993).

Genez, A.L.C.: Cloning and molecular characterization of b-ketoacyl-[ACP] synthases from endosperm of *Ricinus communis*. Genbank # L13241 and # L13242 (1993)

Gland, A., R. Lichter & H.G. Schweiger: Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* (L.). J. Plant Physiol. 132, 613-617 (1988).

Griffiths, G., A.K. Stobart and S. Stymne: Δ-6- and Δ-12-desaturase activities and phospatidic acid formation in microsomal preparations from the developing cotyledons of common borage (*Borago officinalis*). Biochem. J. 252: 641-647 (1988).

Harris, P. & A.T. James: Effect of low temperature on fatty acid biosynthesis in seeds. Biochim. Biophys. Acta 187, 13-18 (1969).

Hays, D.B., P. Rose, S.R. Abrams and M.M. Moloney: Biological activity of optically pure C-1 altered abscisic acid analogs in *Brassica napus* microspore embryos. J. Plant Growth Regul. 15: 5-11 (1996).

Heape A.M., H. Jugelin, F. Boiron and C. Cassagne (1993): Improved one-dimensional thin-layer chromatographic technique for polar lipids (note). J. Chromatogr. 322: 391-395.

Heise, K.P. & J. Fuhrmann: Factors controlling medium-chain fatty acid synthesis in plastids from *Cuphea* embryos. Prog. Lipid Res. 33, 87-95 (1994).

Heppard, E.P., Kinney A.J., Stecca K.L. and G.H. Miao: Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. Plant Physiol. 110: 311-319 (1996).

Hlousek-Radojcic, A., H. Imai and J.G. Jaworski: Oleoyl-CoA is not an immediate substrate for fatty acid elongation in developing seeds of *Brassica napus*. Plant J. 8: 803-809 (1995).

Holbrook, L.A., J.R. Magus and D.C. Taylor: Abscisic acid induction of elongase activity, biosynthesis and accumulation of very long chain monounsaturated fatty acids and oil body proteins in microspore-derived embryos of *Brassica napus* L cv Reston. Plant Sci. 84: 99-115 (1992).

Hugly, S., L. Kunst, J. Browse and C. Somerville: Enhanced thermal tolerance of photosynthesis and altered chloroplast ultrastructure in a mutant of Arabidopsis deficient in lipid desaturation. Plant Physiol. 90: 1134-1142 (1989).

Hugly, S., L. Kunst, J. Browse and C. Somerville: Enhanced thermal tolerance of photosynthesis and altered chloroplast ultrastructure in a mutant of Arabidopsis deficient in lipid desaturation. Plant Physiol. 90: 1134-1142 (1989).

Imai, H., I. Nishida and N. Murata: Acyl-(acyl-carrier protein) hydrolase from squash cotyledons specific to long-chain fatty acids - Purification and characterization. Plant Mol. Biol. 20: 199-206 (1992).

James, D.W., E. Lim, J. Keller, I. Plooy, E. Ralston and H.K. Dooner: Directed tagging of the *Arabidopsis* fatty acid elongation 1 (FAE1) gene with the maize transposon activator. Plant Cell 7: 309-319 (1995).

Jones, A.L., J.E. Dancer and J.L. Harwood: Effect of thiolactomycin on fatty acid synthesis in peas. Phytochemistry 39: 511-514 (1995).

Jönsson, R.: Erucic-acid heredity in rapeseed (*Brassica napus* L. and *Brassica campestris* L.). Hereditas 86: 159-170 (1977).

Jung, J. and K. Grossmann: Effectiveness of new terpenoid derivatives, abscisic acid and its methyl ester on transpiration and leaf senescence of barley. J. Plant Physiol. 121: 361-367 (1985).

Katavic, V., D.W. Reed, D.C. Taylor, E.M. Giblin, D.L. Barton, J.T. Zou, S.L. Mackenzie, P.S. Covello and L. Kunst: Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. Plant Physiol. 108: 399-409 (1995).

Kater, M.M., G.M. Koningstein, H.J.J. Nijkamp and A.R. Stuitje: The use of a hybrid genetic system to study the functional relationship between prokaryotic and plant multi-enzyme fatty acid synthetase complexes. Plant Mol. Biol. 25: 771-790 (1994).

Kauppinen, S.: Structure and expression of the Kas12 gene encoding a b-ketoacyl-acyl carrier protein synthase-I isozyme from barley. J. Biol. Chem. 267: 23999-24006 (1992).

Kim, B.T., Y.K. Min, T. Asami, N.K. Park, I.H. Jeong, K.Y. Cho and S.Yoshida: Synthesis and biological activities of new fluorinated abscisic acid. Bioorg. Med. Chem. Lett. 5: 275-278 (1995).

Kim, K.-S., E. Davelaar and G.-J. de Klerk: Abscisic acid controls dormancy development and bulb formation in lily plantlets regenerated *in vitro*. Physiol. Plant. 90, 59-64 (1994).

Knutzon, D.S, J.L. Bleibaum, J. Nelsen, J.C. Kridl and G.A. Thompson: Isolation and characterization of 2 safflower oleoyl-acyl carrier protein thioesterase cDNA clones. Plant Physiol. 100: 1751-1758 (1992b).

Knutzon, D.S., G.A. Thompson, S.E. Radke, W.B. Johnson, V.C. Knauf and J.C. Kridl: Modifiation of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. Proc. Natl Acad. Sci. 89: 2624-2628 (1992a).

Kocsis, M.G., Weselake R.J., Eng J.A., Furukawa-Stoffer T.L. & M.K. Pomeroy: Phosphatidate phosphatase from developing seeds and microspore-derived cultures of *Brassica napus*. Phytochemistry 41: 353-363 (1996).

Kodama, H., T. Hamada, G. Horiguchi, M. Nishimura and K. Iba: Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco. Plant Physiol. 105: 601-605 (1994).

Koornneef, M., G. Reuling, and C.M. Karssen: The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol. Plant. 61, 377-383 (1984).

Kruger, W.: Root collar and stem rot of swede rape, caused by *Phoma lingam* (stat. gen. *Leptosphaeria maculans*), a disease which is difficult to control. Z. Pflanzenkr. Pflanzenschutz 89: 498-507 (1982).

Kruijff, B. de, W.J. Gerritsen, A. Oerlemans, R.A. Demel and L.L.M. van Deenen: Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlowii* cells and lecithin liposomes. I. Specificity of the permeability changes induced by the polyene antibiotics. Biochim. Biophys. Acta 339: 30-43 (1974)

Laryea, M.D., Y.F. Jiang, G.L. Xu and I. Lombeck: Fatty acid composition of blood lipids in Chinese children consuming high erucic acid rapeseed oil. Ann. Nutrit. Metab. 36: 273-278 (1992).

Lassner, M.W., C.K. Levering, H.M. Davies and D.S. Knutzon: Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil. Plant Physiol. 109: 1389-1394 (1995).

Lassner, M.W., Lardizabal K. and J.G. Metz: A jojoba ß-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell 8: 281-292 (1996).

Little, D., R. Weselake, K. Pomeroy, T Furukawa-Stoffer and J. Bagu: Solubilization and characterization of diacylglycerol acyltransferase from microspore-derived cultures of oilseed rape. Biochem. J. 304: 951-958 (1994).

Martinez-Rivas, J.M., N.C. Thomas, D. Chase, T. Fawcett and A.R. Slabas: cDNA cloning and overexpression of 3-oxoacyl-ACP reductase from *Brassica napus* seed. Grasas Aceites 44: 119-120 (1993).

Millar, A. and L. Kunst: The products of the microsomal fatty acid elongase are determined by the expression and specificity of the condensing enzyme. 12th Int. Symposium on Plant Lipids, Toronto, A15 (1996).

Miquel, M.F., and J.A. Browse: High-oleate oilseeds fail to develop at low temperature. Plant Physiol. 106: 421-427 (1994).

Möllers, C., S. Albrecht & G. Röbbelen: Effect of in vitro culture conditions on fatty acid desaturation in microspore-derived embryoids of *Brassica napus*. J. Plant Physiol. 143, 530-533 (1994).

Murphy D.J. (1988): A highly active soluble diacylglycerol synthesizing system from developing rapeseed, *Brassica napus* L. Lipids 23: 157-163.

Nashaat, N.I. and C.J. Rawlinson: The response of oilseed rape (*Brassica napus* ssp. *oleifera*) accessions with different glucosinolate and erucic acid contents to four isolates of *Peronospora parasitica* (downy mildew) and the identification of new sources of resistance.. Plant Pathol. 43: 278-285 (1994).

Nishida, I., T. Beppu, T. Matsuo and N. Murata: Nucleotide sequence of a cDNA clone encoding a precursor to stearoyl-(acyl-carrier-protein) desaturase from Spinach, *Spinacia oleracea*. Plant Mol. Biol. 19: 711-713 (1992).

Ohlrogge, J. and J. Browse: Lipid biosynthesis. Plant Cell 7: 957-970 (1995).

Okuley, J., J. Lightner, K. Feldmann, N. Yadav, E. Lark and J. Browse: Arabidopsis fad2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6: 147-158 (1994).

Oo, K.C. and A.H.C. Huang: Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. Plant Physiol. 91: 1288-1295 (1989).

Pollard, M.P. and P.K. Stumpf: Biosynthesis of C20 and C22 fatty acids by developing seeds of *Limnanthes alba*. Plant Physiol. 66, 649-655 (1980b).

Pollard, M.P. and P.K. Stumpf: Long Chain (C20 and C22) Fatty acid biosynthesis in developing seeds of *Tropaeolum majus*. Plant Physiol. 66, 641-648 (1980a).

Pomeroy, K., D.C.W. Brown and Y. Takahata: Response of *Brassica napus* L. microspore-derived embryos to exogenous abscisic acid and desiccation. In Vitro Cell. Dev. Biol. - Plant 30P, 196-203 (1994).

Post-Beittenmiller, D., J.G. Jaworsji and J.B. Ohlrogge: In vivo pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J. Biol. Chem. 266: 1858-1865 (1991).

Post-Beittenmiller, D.: Biochemistry and molecular biology of wax production in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 405-430 (1996).

Rawlinson, C.J., B.C. Sutton and G. Muthyalu: Taxonomy and biology of *Pyrenopeziza brassicae* sp. nov. (*Cylindrosporium concentricum*), a pathogen of winter oilseed rape (*Brassica napus* spp. *oleifera*). Trans. Br. Mycol. Soc. 71: 425-439 (1978).

Rose, P.A., B. Lei, A.C. Shaw, M.K. Walker-Simmons, S. Napper, J.W. Quail and S.R. Abrams: Chiral synthesis of (+)-8'-demethyl abscisic acid. Can. J. Chem. 74: 1836-1843 (1996).

Ross, G.S., P.A. Elder, J.A. McWha, D. Pearce and R.P. Pharis: The development of an indirect enzyme linked immunoassay for abscisic acid. Plant Physiol. 85, 46-50 (1987).

Scarth, R., P.B.E. McVetty and S.R. Rimmer: Mercury high erucic low glucosinolate summer rape. Can. J. Plant Sci. 75: 205-206 (1995).

Schneider, F., R. Lessire, J.J. Bessoule, H. Juguelin and C. Cassagne: Effect of cerulenin on the synthesis of very-long-chain fatty acids in microsomes from leek seedlings. Biochim. Biophys. Acta 1152: 243-252 (1993).

Schuch, R., M. Brummel & F. Spener: Medium-chain acyl-ACP thioesterase is not the exclusive enzyme responsible for early chain-length termination in medium-chain fatty acid synthesis. Grasas Aceites 44, 126-128 (1993).

Schuch, R., M. Brummel and F. Spener: ß-Ketoacyl-acyl carrier protein (ACP) synthase III in *Cuphea lanceolata* seeds: Identification and analysis of reaction products. J. Plant Physiol. 143: 556-560 (1994).

Schuster, W. & A. Taghizadeh: Über Leistungen und Qualität von Sommerraps auf ökologisch stark differenzierten Standorten. Bayerisches Landw. Jahrbuch 57, 220-237 (1980).

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

Shintani, D., and J. Ohlrogge: Feedback inhibition of fatty acid synthesis in tobacco suspension cells. Plant J. 7: 577-587 (1995).

Singal, H.R., G. Talwar, A. Dua and R. Singh: Pod photosynthesis and seed dark CO_2 fixation support oil synthesis in developing *Brassica* seeds. J. Biosci. 20, 49-58 (1995).

Slabaugh, M.B., H. Tai, J. Jaworski and S.J. Knapp: cDNA clones encoding ß-ketoacyl-acyl carrier protein synthase III from *Cuphea wrightii*. Plant Physiol. 108: 443-444 (1995).

Sleiman, A.I., B. Boelcke and M. Seiffert: Studies on the germination behaviour of winter rape (*Brassica napus* L.) under the influence of growth regulators. Untersuchungen zum

Keimungsverhalten von Winterraps (*Brassica napus* L.) unter dem Einfluss von Wachstumsregulatoren. Wiss. Z. Wilhelm-Pieck-Univ. Naturwiss. Reihe 36: 105-107 (1987).

Slocombe, S.P., I. Cummings, R.P. Jarvis and D.J. Murphy: Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol. Biol. 20: 151-155 (1992).

Stryer, L.: Biochemistry, 2nd edition. Freeman and Co., New York, USA (1981).

Suttle, J.C. and S.R. Abrams: Abscission-promoting activities of abscisic acid and five abscisic acid analogs in cotton seedlings and explants. Plant Growth Regul. 12: 111-117 (1993).

Tai, H.Y. and J.G. Jaworski: 3-Ketoacyl-acyl carrier protein synthase-III from spinach (*Spinacia oleracea*) is not similar to other condensing enzymes of fatty acid synthase. Plant Physiol. 103: 1361-1367 (1993).

Tai, H.Y., D. Post-Beittenmiller and J.G. Jaworski: Cloning of a cDNA encoding 3-ketoacyl-acyl carrier protein synthase III from Arabidopsis. Plant Physiol. 106: 801-802 (1994).

Taylor, D.C. and N. Weber: Microspore-derived embryos of the *Brassicaceae* - model system for studies of storage lipid bioassembly and its regulation. Fat Sci. Technol. 96, 228-235 (1994).

Taylor, D.C., D.L. Barton, E.M. Giblin, S.L. Mackenzie, C.G.J. Vandenberg and P.B.E. Mcvetty: Microsomal lyso-phosphatidic acid acyltransferase from a *Brassica oleracea* cultivar incorporates erucic acid into the sn-2 position of seed triacylglycerols. Plant Physiol. 109: 409-420 (1995).

Taylor, D.C., D.L. Barton, K.P. Rioux, S.L. Mackenzie, D.W. Reed, E.W. Underhill, M.K. Pomeroy and N. Weber: Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv Reston. Plant Physiol. 99: 1609-1618 (1992).

Taylor, D.C., N. Weber, D.L. Barton, E.W. Underhill, L.R. Hogge, R.J. Weselake and M.K. Pomeroy: Triacylglycerol bioassembly in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Physiol. 97, 65-79 (1991).

Taylor, D.C., N. Weber, E.W. Underhill, M.K. Pomeroy, W.A. Keller, W.R. Scowcroft, R.W. Wilen, M.M. Moloney & L.A. Holbrook: Storage-protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta 181, 18-26 (1990).

Töpfer, R. and N. Martini: Molecular cloning of cDNAs or genes encoding proteins involved in *de novo* fatty acid biosynthesis in plants. J. Plant Physiol. 143: 416-425 (1994).

Tremolières, A., J.P. Dubacq & D. Drapier: Unsaturated fatty acids in maturing seeds of sunflower and rape: regulation by temperature and light intensity. Phytochemistry 21, 41-45 (1982).

USDA (USA Dept. of Agriculture): Oilseeds and products: World markets and trade, circular October 1996, Washington (1996)

Verwoert, I.I.G.S., A. Brown, A.R. Slabas and A.R. Stuitje: A *Zea mays* GTP-binding protein of the ARF family complements an *Escherichia coli* mutant with a temperature-sensitive malonyl-coenzyme A:acyl carrier protein transacylase. Plant Mol. Biol. 27: 629-633 (1995a).

Verwoert, I.I.G.S., K.H. van der Linden, M.C. Walsh, H.J.J. Nijkamp and A.R. Stuitje: Modification of *Brassica napus* seed oil by expression of the *Escherichia coli* fabH gene, encoding 3-ketoacyl-acyl carrier protein synthase III. Plant Mol. Biol. 27: 875-886 (1995b).

Voelker, T.A., T.R. Hayes, A.M. Cranmer, J.C. Turner and H.M. Davies: Genetic engineering of a quantitative trait: Metabolic and genetic parameters influencing the accumulation of laurate in rapeseed. Plant J. 9: 229-241 (1996).

Vollmann, J. & P. Ruckenbauer: Agronomic performance and oil quality of Crambe as affected by genotype and environment. Bodenkultur 44, 335-343 (1993).

Vonk, C.R., E. Davelaar and S.A. Ribot; The role of cytokinins in relation to flower bud blasting in *Iris* cv. Ideal: cytokinin determination by an improved enzyme-linked immunosorbent assay. Plant Growth Regul. 4, 65-74 (1986).

Walker-Simmons, M.K., P.A. Rose, A.C. Shaw and S.R. Abrams: The 7'-methyl group of abscisic acid is critical for biological activity in wheat embryo germination. Plant Physiol. 106: 1279-1284 (1995).

Walker-Simmons, M.K., R.J. Anderberg, P.A. Rose and S.R. Abrams: Optically pure abscisic acid analogs - tools for relating germination inhibition and gene expression in wheat embryos. Plant Physiol. 99: 501-507 (1992).

White, C. and C. Rivin: Overlapping pathways of ABA-modulated gene expression during embryo maturation. Maize Genet. Coop. Newsletter 67: 37 (1993).

Wilen, R.W., D.B. Hays, R.M. Mandel, S.R. Abrams and M.M. Moloney: Competitive inhibition of abscisic acid-regulated gene expression by stereoisomeric acetylenic analogs of abscisic acid. Plant Physiol. 101: 469-476 (1993).

Xu, N.F., and J.D. Bewley : The role of abscisic acid in germination, storage protein synthesis and desiccation tolerance in alfalfa (*Medicago sativa* L) seeds, as shown by inhibition of its synthesis by fluridone during development. J. Exp. Bot. 46, 687-694 (1995).

Yukawa, Y., F. Takaiwa, K. Shoji, K. Masuda and K. Yamada: Structure and expression of two seed-specific cDNA clones encoding stearoyl-acyl carrier protein desaturase from sesame, *Sesamum indicum* L. Plant Cell Physiol. 37: 201-205 (1996).

Summary

Vegetable oils are an important commodity world-wide with an annual production of about 70 million tonnes. Oilseed rape is one of the four major crops, providing about 10% of the total production. Quality of vegetable oils is determined by the fatty acid composition of the triacylglycerols (TAG) that constitute such oils. These fatty acids comprise a range of chain lengths and desaturated and oxidised residues. A small group of fatty acids dominates the edible oils which are the predominant products, whereas other specific types of fatty acids are used in specialised applications and generally occur in small crops and wild species. One of these fatty acids is erucic acid (22:1), a very long chain monounsaturated fatty acid, which naturally occurs in species of the *Brassicaceae*.

Synthesis of all fatty acids requires a group of enzymes, most of which have been isolated from a number of species. After synthesis of the primary fatty acids, palmitic (16:0), stearic (18:0) and oleic acid (18:1), in the plastids, they are exported as their CoA-esters into the cytoplasm, where modification and incorporation in lipids occurs. This requires another group of enzymes, some of which, like desaturases and acyltransferases, have been isolated and characterised but most of the species specific modifying enzymes have not been isolated yet. However, little is known about the mechanisms that regulate and coordinate the expression and activity of these enzymes. This thesis focuses on the accumulation of 22:1 and the regulation of the elongase synthesising this fatty acid from 18:1.

It has been reported that 22:1 levels can be influenced by growth temperature (Canvin, 1965) but little was known about the regulatory mechanism and timing of this influence. We compared the accumulation of oil and 22:1 in seeds of different cultivars at two temperatures, 15 and 25 °C. It was shown that the level of 22:1 in seeds increases from 30 to 40 mol% with a temperature

decrease from 25 to 15 °C in only one cultivar, Reston, whereas Gulle was shown to be insensitive to changes in temperature. In these experiments we also showed that growth temperature exerts its effect only during the time of maximum oil synthesis, not before or after.

Similar experiments showed that microspore-derived embryos (MDEs), grown *in vitro*, followed a similar pattern of oil accumulation and timing of temperature influence as described for seeds on the intact plant. However, the absolute levels of oil and 22:1 were much lower in MDEs than in seeds and the fraction 22:1 of total fatty acids was reduced by about 10 mol% in MDEs.

Analysis of the levels of abscisic acid (ABA) in developing seeds together with the low levels of this plant growth regulator in MDEs suggested that ABA may be an important factor in determining the level of 22:1 in the oil of oilseed rape. However, dose response curves for ABA in MDEs grown at 15 and 25 °C showed that the sensitivity to ABA is not influenced by culture temperature. At both temperatures a 50% response was observed at about 0.3 μM and the increase in 22:1 was about 10 mol% at saturating ABA levels. We also found that ABA levels in seeds are saturating at both temperatures, implying that ABA cannot be an intermediate in the transduction of a temperature signal.

In addition, statistical analysis of temperature and ABA effects in MDEs showed no significant interaction between these two stimuli. This was further confirmed by the fact that, absolute amounts of both oil and 22:1 increased upon addition of ABA, whereas with temperature only affected the fraction of 22:1 and not the total amount of oil in MDEs.

We also studied the effects of a group of demethyl-ABA analogs. In this study we found that the 7'-methyl group is very important for ABA activity. Removal of this group resulted in a 100-fold increase in the amount of the (+)-enantiomer needed to induce a similar increase in 22:1 accumulation as compared to natural (+)-ABA while a complete loss of activity was observed for (-)-7'-demethyl-ABA. The function of the 8'- and 9'-methyl groups is less clear.

Removal of these groups resulted in a partial reduction in ABA activity, but the effects were different for total fresh weight accumulation and 22:1 levels. This suggests that at least two types of ABA receptors operate in MDEs.

Changes in 22:1 level in the oil must be caused by changes in the enzyme activities catalysing oil biosynthesis. Holbrook *et al.* (1992) had already shown that the addition of ABA to culture medium resulted in an increase in elongase activity, but little was known about the effect of temperature on elongase activity. We elaborated on the effects of ABA application on elongase activity in MDEs at two different temperatures, 15 and 25°C. We found higher total elongase activities in MDEs grown at 15 °C, but temperature sensitivity and effect of 18:1-CoA concentration were not affected.

The differences in total activity correlated closely with the differences observed in 22:1 amount in the MDEs, suggesting that the amount of 22:1 is regulated by the total amount of elongase activity. The correlation between elongase activity and the fraction 22:1 in the oil was lower and no correlation was found between acyltransferase activity and the amount of oil accumulated. It was not possible to properly determine the kinetic parameters K_m and V_{max} of the elongase complex due to rapidly loss of activity upon isolation from the membrane and to substrate inhibition by 18:1-CoA at relatively low concentrations (about 10 µM). This inhibition by 18:1-CoA is apparently caused by a detergent effect of this compound disrupting the membrane in which the elongase complex is embedded.

Based on these observations a model was formulated to describe the regulation of 22:1 accumulation and oil composition in oilseed rape. The total amount of oil is regulated by the activity of fatty acid synthase (FAS), synthesising fatty acids from acetyl-CoA, derived from carbohydrates imported into the embryo. The main product of FAS, oleic acid, is exported from the plastid and enters the cytoplasm as 18:1-CoA. The pool of 18:1-CoA is modified by desaturases and elongase transforming part of it into other fatty acids like 18:2 and 22:1.

Subsequently, the fatty acids in this pool are incorporated in TAG. The acyltransferases performing two out of three of these reactions have little or no selectivity for the various fatty acids and therefore the fatty acid composition of the oil largely reflects the fatty acid composition of the acyl-CoA pool. The fraction of 22:1 in this pool is determined by the relative activity of the elongase in comparison to the total flux of fatty acids through the pool.

With the knowledge that elongase activity regulates the amount of 22:1, it must be possible to increase the level of 22:1 in the oil beyond the level of about 55% observed so far (Scarth *et al.*, 1995). Selectivity of the second acyltransferase, excluding 22:1 from the sn-2 position of TAG, which would limit the maximum 22:1 level to 67%, has been circumvented by transformation of oilseed rape with an acyltransferase that can incorporate 22:1 at this position (Lassner *et al.*, 1995). Whether it will be possible to increase the level of 22:1 in the oil of oilseed rape up to 90 or 100% of fatty acids, greatly increasing the value of rapeseed oil for industrial purposes, will depend upon the effects of increased 22:1 levels on lipid metabolism.

Samenvatting

Plantaardige oliën zijn een belangrijk produkt op de wereldmarkten met een jaarlijkse omzet van ongeveer 70 miljoen ton. Koolzaad is een van de vier belangrijkste oliegewassen met een produktie van bijna 10% van de totale hoeveelheid. De kwaliteit van plantaardige olie hangt vooral af van de vetzuursamenstelling van de triacylglycerolen (TAG), de moleculen waaruit de olie voornamelijk bestaat. Er is een grote verscheidenheid aan vetzuren in planten, met variatie in ketenlengte, mate van onverzadigdheid en de aanwezigheid van allerlei zuurstofhoudende groepen. Slechts een kleine groep van al deze vetzuren wordt gebruikt in voedingsmiddelen, de grootste toepassing van plantaardige olie, alle andere worden voor speciale toepassingen gebruikt en komen vooral voor in kleine gewassen en wilde plantensoorten. Erucazuur (22:1), een zeer langketenig, enkelvoudig onverzadigd vetzuur is een voorbeeld van een dergelijk vetzuur dat voorkomt in soorten van de familie *Brassicaceae*.

Voor de biosynthese van vetzuren is een groep enzymen nodig waarvan de meeste reeds uit een of meer plantensoorten zijn geïsoleerd. Nadat de primaire vetzuren palmitinezuur (16:0), stearinezuur (18:0) en oliezuur (18:1) in de plastide zijn gevormd worden ze getransporteerd naar het cytoplasma, waar ze kunnen worden gemodificeerd en ingebouwd in lipiden. Hiervoor is weer een andere groep enzymen noodzakelijk waarvan sommige, zoals desaturases en acyltransferases, zijn geïsoleerd en gekarakteriseerd. Over de meeste andere vetzuur-modificerende enzymen is nog weinig bekend. Nog minder weten we over het mechanisme dat de expressie en coördinatie van al deze verschillende enzymen reguleert. In dit proefschrift wordt ingegaan op de accumulatie van 22:1 en op de regulatie van het elongase, het enzym dat 22:1 maakt uit 18:1.

In 1965 werd aangetoond dat het 22:1-gehalte in de olie kan worden beïnvloed door de omgevingstemperatuur (Canvin, 1965), maar op welk moment deze

invloed optrad was niet duidelijk. Wij hebben bestudeerd wat er gebeurt met het 22:1-gehalte in zaden van verschillende koolzaadrassen bij twee temperaturen, 15 en 25 °C. In het ras Reston was bij de laagste van deze twee temperaturen het 22:1-gehalte ongeveer 10% hoger dan bij de hogere temperatuur (respectievelijk 30 en 40% 22:1) terwijl in een ander ras, Gulle, geen verschillen konden worden aangetoond. In deze zelfde experimenten werd tevens aangetoond dat deze temperatuursinvloed alleen optrad in de periode dat de olie wordt gevormd, niet er voor of er na.

Vergelijkbare experimenten werden ook gedaan met microspore-afgeleide embryo's (MDEs), waarin hetzelfde patroon van 22:1 accumulatie en temperatuursinvloed werd waargenomen als in zaden. Er werd echter minder olie en 22:1 aangetoond in MDEs en het 22:1-gehalte was ongeveer 10% lager dan in zaden.

Bepaling van het gehalte aan abscissinezuur (ABA) gedurende de ontwikkeling van zaden en het feit dat deze plantengroeistof slechts in heel lage concentratie werd aangetoond in MDEs suggereerden dat ABA een belangrijke factor kan zijn die mede het 22:1-gehalte in koolzaad bepaalt. Daarom hebben wij dosis-respons curven gemaakt voor ABA in MDEs gekweekt bij 15 en 25 °C, waaruit naar voren kwam dat de kweektemperatuur geen invloed heeft op de gevoeligheid voor ABA; bij beide temperaturen werd een 50% respons gevonden bij ongeveer 0.3 µM ABA en was het 22:1-gehalte 10% hoger bij verzadigende ABA-concentraties dan bij de controle. Tevens bleek dat de in zaden gevonden ABA-gehalten in het verzadigende gebied van de dosis-respons curve liggen en dat ABA dus geen stap kan zijn in de doorgifte van een temperatuursignaal.

Tenslotte toonde statistische analyse geen significante interactie tussen temperatuur- en ABA-behandelingen in MDEs. Dit werd bevestigd door het feit dat de absolute hoeveelheden olie en 22:1 toenamen bij ABA toediening, terwijl

bij temperatuurverandering de hoeveelheid olie hetzelfde bleef in MDEs, en alleen het 22:1 gehalte veranderde.

Ook is de invloed van methylgroepen aan de ring van ABA bestudeerd. In deze studie bleek dat de 7'-methyl groep erg belangrijk is om de activiteit van ABA te behouden. Bij toediening van (+)-7'-demethyl-ABA is een 100-voudige concentratie nodig om het in dezelfde mate te 22:1-gehalte te verhogen als met natuurlijk (+)-ABA, terwijl(-)-7'-demethyl-ABA volledig inactief is. De functie van de 8'- en 9'-methyl groepen is minder helder. Als deze groepen van het molecuul worden verwijderd, wordt de ABA activiteit minder, maar de effecten op groei en 22:1-gehalte zijn niet consistent. Dit suggereert dat ABA betrokken is bij tenminste twee verschillende signaalroutes in MDEs.

Veranderingen in het 22:1-gehalte in de olie kunnen slechts ontstaan door veranderingen in de enzym-activiteiten die oliesynthese katalyseren. Holbrook *et al.* (1992) hadden al aangetoond dat elongase-activiteit wordt verhoogd door toediening van ABA aan MDEs, maar effecten van kweektemperatuur op elongase-activiteit waren nog niet bestudeerd. Wij zijn dieper ingegaan op de effecten van ABA toediening op elongase activiteit bij twee verschillende temperaturen, 15 en 25 °C. In deze studie werden verschillen waargenomen in totale elongase-activiteit, maar niet in temperatuurgevoeligheid of afhankelijkheid van 18:1-CoA concentratie, tussen de enzympreparaten uit MDEs gekweekt onder de verschillende condities.

De verschillen in totale activiteit tonen een nauwe correlatie met de verschillen die optreden in de absolute hoeveelheid 22:1 in MDEs, wat suggereert dat de hoeveelheid 22:1 wordt gereguleerd door de hoeveelheid elongase activiteit. De correlatie tussen elongase activiteit en het 22:1-gehalte in de olie was minder duidelijk en er was geen correlatie tussen acyltransferase activiteit en de hoeveelheid olie die werd gevormd in MDEs. Het was niet mogelijk om K_m- en V_{max}-waarden te bepalen omdat het enzymcomplex zijn activiteit verliest als het uit de membraan wordt geïsoleerd en omdat substraatremming optreedt

door 18:1-CoA bij relatief lage concentraties (ongeveer 10 μM). De remming door 18:1-CoA wordt waarschijnlijk veroorzaakt door een detergentwerking van deze stof waardoor de membraan waarin het elongase is ingebed wordt verstoord.

Op basis van deze waarnemingen is een model geformuleerd dat beschrijft hoe de 22:1-accumulatie en oliesamenstelling worden gereguleerd. De totale hoeveelheid olie wordt bepaald door de activiteit van het vetzuursynthase complex (FAS), dat vetzuren maakt uit acetyl-CoA wat weer gesynthetiseerd wordt uit suikers. Het belangrijkste produkt van het FAS, 18:1, wordt dan in de vorm van 18:1-CoA uit de plastide naar het getransporteerd. Een deel van het 18:1-CoA kan dan worden omgezet door desaturase- en elongaseactiviteit in 18:2, 18:3, 20:1 en 22:1, waarna de vetzuren worden ingebouwd in TAG. Twee van de drie acyltransferases betrokken bij TAG-synthese zijn niet selectief voor specifieke vetzuren, en de vetzuursamenstelling in TAG weerspiegelt dan ook in belangrijke mate de samenstelling van de groep vetzuren in het cytoplasma. Het 22:1-gehalte in deze groep vetzuren wordt bepaald door de verhouding tussen de elongase-activiteit en de totale vetzuur-flux door het cytoplasma.

Nu we weten dat de elongase-activiteit de hoeveelheid 22:1 reguleert moet het mogelijk zijn om het 22:1-gehalte in koolzaadolie te verhogen boven het tot nu toe hoogste niveau van ongeveer 55% (Scarth et al., 1995). Het is al mogelijk gebleken om het acyltransferase dat op de sn-2 positie vetzuren inbouwt, en dat in koolzaad geen 22:1 accepteert waardoor het maximum 22:1-gehalte wordt beperkt tot 67%, langs moleculair-biologische weg te vervangen door een acyltransferase dat dit vetzuur wel accepteert (Lassner *et al.*, 1995). Of het praktisch mogelijk is om het 22:1-gehalte in koolzaadolie te verhogen tot 90-100%, wat de waarde van koolzaadolie als industriële grondstof sterk zal verhogen, moet nog worden afgewacht en zal afhangen van de effecten van zulke hoge 22:1-gehalten op het lipiedmetabolisme.

Curriculum vitae

Jeroen A. Wilmer werd op 22 juni 1966 geboren te Bergen op Zoom. In 1984 werd de VWO opleiding voltooid aan het Gymnasium Beekvliet te Sint Michielsgestel. In datzelfde jaar begon hij aan een studie plantenveredeling aan de Landbouwuniversiteit, toen nog Landbouwhogeschool. In januari 1990 werd het doctoraal examen behaald met als afstudeervakken moleculaire biologie en plantenfysiologie. Na voltooiing van de militaire dienst heeft hij in 1992 enkele maanden op de vakgroep plantenfysiologie gewerkt aan een na-doctoraal onderzoeks project (NOP) op het gebied van uitdroogtolerantie in zaden alvorens op 1 juni 1992 in dienst te treden van deze vakgroep als AiO. Hij werd gedetacheerd op het AB-DLO, alwaar hij tot december 1996 heeft gewerkt aan het onderzoek dat in dit proefschrift staat beschreven.