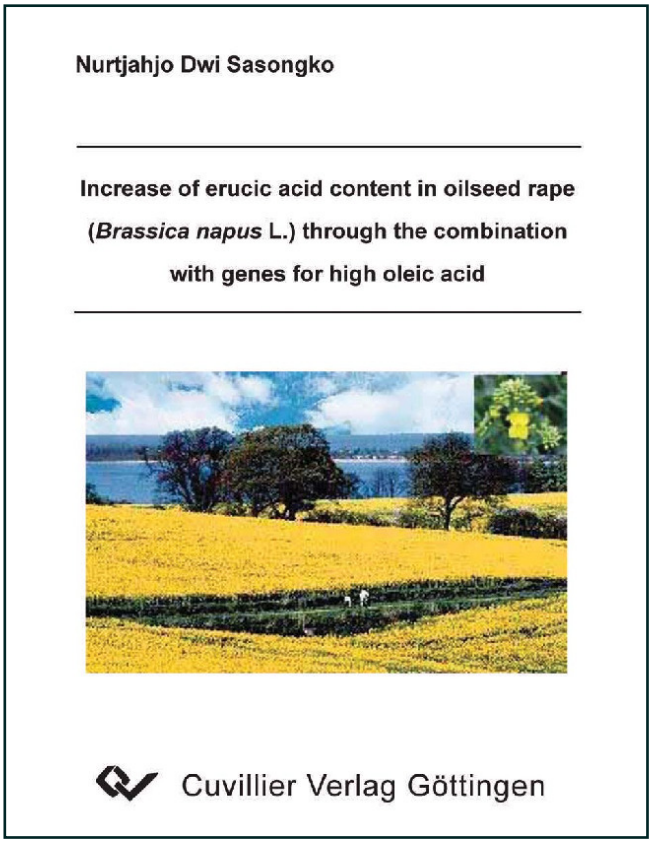
Nurtjahjo Dwi Sasongko (Autor)

Increase of erucic acid content in oilseed rape (Brassica napus L.) through the combination with genes for high oleic acid



https://cuvillier.de/de/shop/publications/3260

1 Introduction

1.1 Importance of rapeseed oils

Rapeseed (*Brassica napus* L.) is one among several important plants used as source of vegetable oils. At present, rapeseed oil is following soybean and palm oil in world production and is used for both nutritional and industrial purposes (Piazza and Foglia, 2001, Walker and Booth, 2001). Nutritionally, replacement of saturated fats/oil with unsaturated ones contained in vegetable oils on the daily diet could help to prevent from coronary heart disease (Freese, 2001), mainly by lowering serum cholesterol levels (see Velasco et al., 1999d). Fast growing demands of rapeseed oil as food source, therefore, is related to increased consciousness of healthy food (Murphy, 1994). Industrial use of vegetable oils, however, is more related to its different fatty acid contents. Sonntag (1995) stated that behenic acid (C22:0), and erucic acid (C22:1) have been applied substantially in the oleochemical industry.

In traditional *Brassica* oilseeds, the occurence of two components C22:1 and glucosinolates (GSL) traditionally distinguishes them from other major oil seeds (Lühs and Friedt, 1994). Since these two components are considered as antinutritional for both human and animals their contents were minimized by traditional breeding, which finally resulted in the release of Canola or '00' quality type. This rapeseed oil contains ≤2% C22:1, and in Europe contains ≤25 μmol/g seed GSL. Furthermore, Canola type rapeseed contains only about 7% saturated fatty acids (palmitic, C16:0+stearic, C18:0) while having about 60% oleic acid (C18:1), 20% linoleic acid (C18:2), and 10% linolenic acid (C18:3). Nowadays, different types of rapeseeds with a modified fatty acid composition are available for different purposes (Table 1.1).

Table 1.1 Fatty acid composition of different rapeseed quality types in % of total fatty acids (Möllers, 2002b)

Oil quality (fatty acids)	Saturated*	Oleic C18:1	Linoleic C18:2	Linolenic C18:3	Erucic C22:1
'00' Canola	7	60	20	10	<2
High Erucic (HEAR)	6	15	13	9	58**
Low Linolenic (LL)	7	60	30	2	<2
High Oleic (HOAR)	5	86	4	4	<2
High Oleic/Low Linolenic (HOLL)	5	85	6	2	<2

* mainly C16:0 (3-4%) and C18:0 (1-2%)
** includes ≈8% C20:1

Apart from their nutritional value, reduction of polyunsaturated fatty acids are important from technical point of view. Oil containing a low content of polyunsaturated fatty acids (C18:2 and C18:3) is more stable at high temperature without smoking, and it is less prone to oxidative changes during refining, storage and frying (Miller et al., 1987, Scarth et al., 1988, Lopez et al., 2000). The first attempt to increase the oil stability was started by developing 'Stellar', a Canadian spring rape cultivar following the success of mutagenesis of the 'Oro' cultivar (Röbbelen and Nitsch, 1975), to produce 'Low Linolenic' (LL, see Table 1.1) type rapeseed.

Tailoring oleic acid content to more than 80% and lowering C18:2 and C18:3 to around 10% for the sum of these fatty acids has been achieved through mutation breeding and gene technology. These types are classified as high oleic acid rapeseed or high oleic/low linolenic rapeseed (HOLL) (Auld et al. 1992, Rücker and Röbbelen, 1995, Stoutjesdijk et. al., 1999, Schierholt and Becker, 2001).

Development of rapeseed containing high erucic acid is also of interest for plant breeders, since this component and its derivatives are important raw material for industrial applications (Piazza and Foglia, 2001). This fatty acid is

known as a major component in *Brassicaceae* and *Tropaeolaceae* and is a valuable renewable, biodegradable, low toxicity, and environmentally friendly resource in the oleochemical industries and is used as: plasticizers, lubricants, slip and coating agents, etc. (see Table 1.3; Sonntag, 1995, Taylor et al., 1995, Piazza and Foglia, 2001). High erucic acid rapeseed (HEAR) was cultivated on 27000 ha in Germany and 55000 ha were grown in Europe in 2001/2002 (personal communication Reisewitz, Raiffeisen Hauptgenossenschaft Nord AG, Hannover, cited in Möllers, 2002a). However, *Brassica* oil with proportion of C22:1 higher than 55% is still being sought by breeders, biotechnologists, and chemists (Lühs and Friedt, 1994).

1.2 Biosynthesis of fatty acids in *Brassica napus* L.

During seed development, the embryo of *Brassica napus* synthesize the seed's reserve materials which consist mainly of lipids and proteins and which are important in supplying nutrition required for germination and juvenile growth. In this process, the seed may accumulate fatty acids with different amount of carbon chain lengths as well as degree of saturation (Cramer, 1990).

The first product of non associated plant fatty acid synthethase is C16:0, elongated by palmitoyl-ACP elongase to stearoyl-ACP (C18:0; Figure 1.1). Due to Δ9 stearoyl-ACP fatty acid desaturase enzyme, C18:0 is then desaturated to C18:1. Palmitic, stearic, and oleic acid may also be released by an acyl-ACP thioesterase and reesterified on the chloroplast envelope to Coenzyme A (C16:0-CoA, C18:0-CoA, and C18:1-CoA; Downey,1987). In *Arabidopsis* as well as in rapeseed, C18:1 is further desaturated by a Δ12-desaturase to form C18:2 and the presence of Δ15-desaturase is needed to desaturase C18:2 to C18:3 (Arondel et al., 1992, Okuley et al., 1994). However, oleic acid in form of C18:1-CoA might also be sequentially elongated first to C20:1 and then to C22:1.

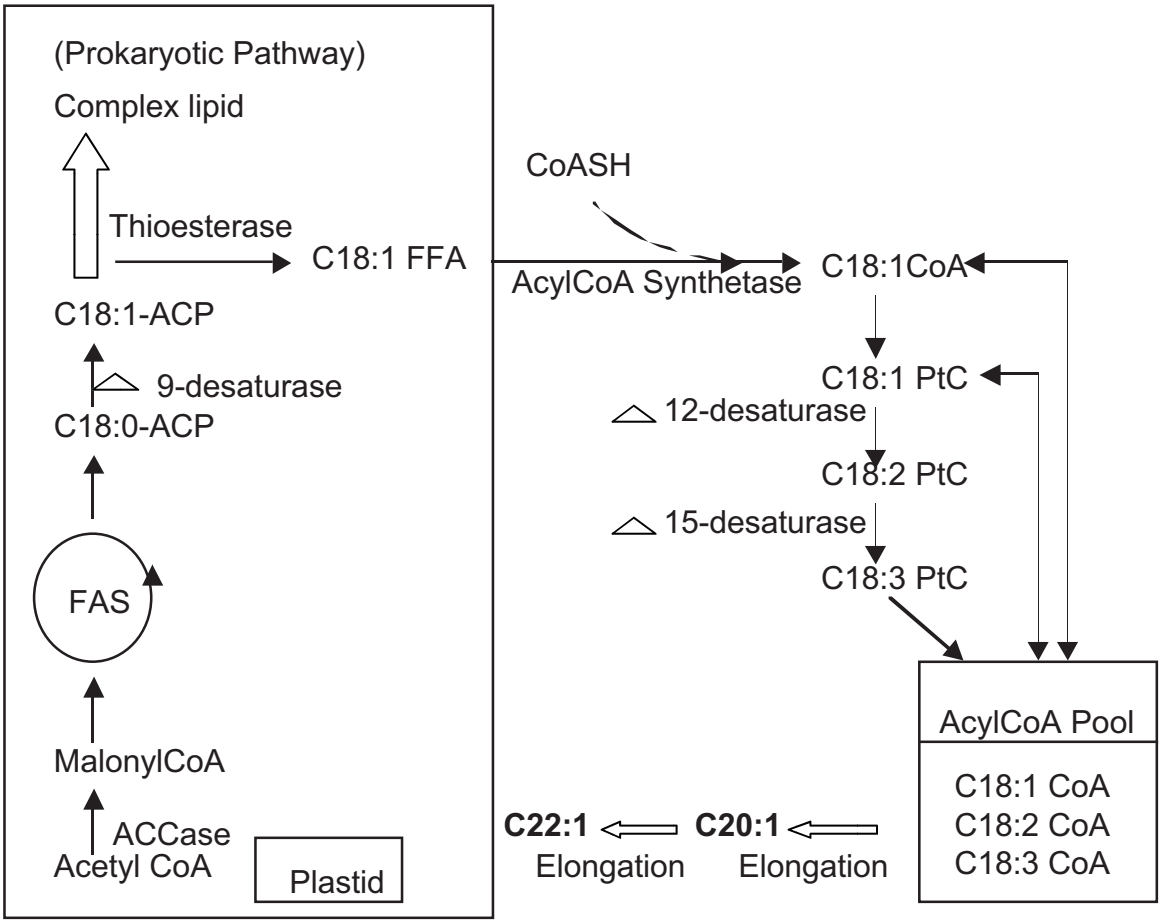


Figure 1.1 A diagram of erucic acid (C22:1) biosynthesis in *B. napus* (L.) adopted from Slabas et al. (2001). ACCase = Acetyl-CoA carboxylase. ACP = Acyl Carrier Protein. FAS = fatty acid synthetase. FFA = free fatty acid. PtC = Phosphatidyl choline. CoASH = Activated CoA.

The elongation process of C18:1 to C22:1 occurs in the cytoplasm and is catalysed by the membrane-bound oleoyl-CoA elongase complex through four successive reactions. As a product of these four reactions, C20:1 is formed, which undergoes the same reactions to yield C22:1 (Cassagne et al., 1994). Stumpf (1980), Millar and Kunst (1997), and Puyaubert et al. (2000) stated that these four steps are as follows (see also Figure 1.2):

a) condensation of malonyl-CoA with a long chain acyl-CoA by a condensing enzyme called β-ketoacyl-CoA synthase (KCS) leads to β-ketoacyl-CoA.

b) reduction of β-ketoacyl-CoA to β-hydroxyacyl-CoA by β-ketoacyl-CoA reductase, which uses NAD(P)H as the reductant

c) dehydration of β-hydroxyacyl-CoA to an enoyl-CoA by β-OH-acyl-CoA dehydratase.

d) enoyl-CoA reduction by *trans*-2,3-enoyl-CoA reductase with NAD(P)H as reductant to the long chain acyl CoA

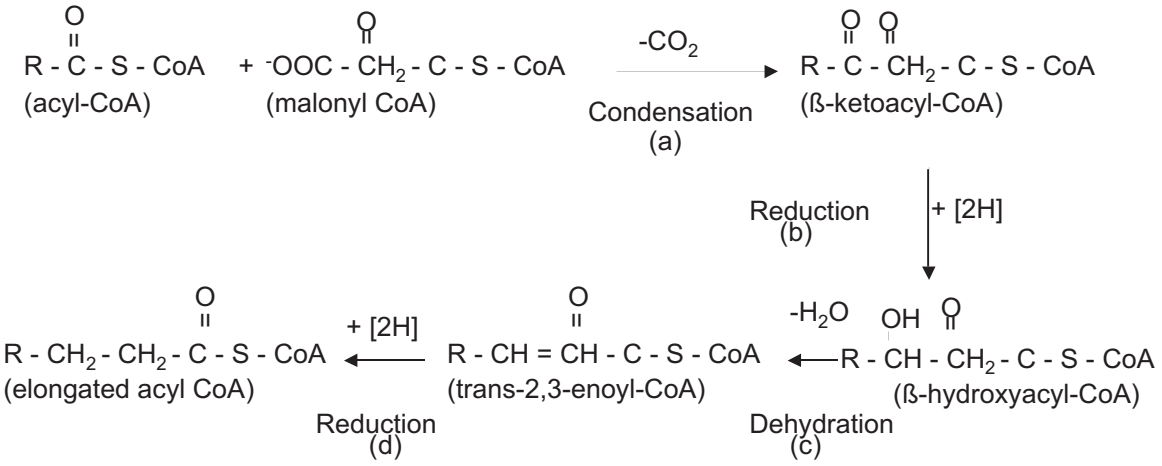


Figure 1.2 Elongation process of Acyl-CoA to eicosenoic and erucic acid in *Brassica napus* (Harwood,1996, Wallis, et al., 2002; a,b,c,d = see text for explanation).

Mechanisms of the elongation process have been studied in various higher plants such as: *A. thaliana*, *Simmondsia chinensis*, *Allium porum*, *Lunaria annua* and *B. napus*. Fehling and Mukherjee (1990) concluded that for the synthesis of C22:1 in oilseed plants, C18:1-CoA and C20:1-CoA are the substrates for this elongase. James et al. (1995) isolated the fatty acid elongase (*fae1*)-gene of *A. thaliana* using transposon tagging which encodes for the condensing enzyme (step a in Figure 1.2). Following to this, a couple of cDNA's encoding putative KCS enzyme have been isolated from different species.

In relation to the condensation process, Millar and Kunst (1997) concluded that the *fae1*-gene encoding the β-ketoacyl-CoA synthase (KCS) is the first

and main limiting factor in the synthesis of erucic acid in developing seeds of *Arabidopsis*. Mutation of this gene resulted in the deficiency of fatty acid elongation from C18:1 to C20:1, and further from C20:1 to C22:1 (Kunst et al., 1992).

1.3 Genetic approaches to modify fatty acid content in rapeseed oil

Tailoring fatty acid composition of rapeseed for desirable fatty acids have been done thoroughly without affecting oil content, seed germination, seedling vigour or even harming the plants (Downey, 1987). Mutagenesis and molecular gene transfer in combination with classical breeding has been applied in the past to most efficiently modify oil quality (Auld et al., 1992, Knapp and Crane, 1998, Lu et al., 2001, Millar and Kunst, 1997, Rücker and Röbbelen, 1995). Spontaneous mutation, for example, has been used to develop '00' quality rapeseed. Mutagenesis has also been applied to develop the LL and HO rapeseed (see Table 1.1).

Using *Arabidopsis* mutants, Lemieux et al. (1990) stated that C18:1 synthesis is affected by a single mutated nuclear gene designated as *fad2* which encodes for the oleic acid desaturase (Figure 1.1). This significantly increased oleic acid from around 15% in the wild type to more than 50%. Miquel and Browse (1994) stated that the synthesis of polyunsaturated fatty acid (PUFA, C18:2 and C18:3) in plants is controlled by the *fad2*-gene. Schierholt et al. (2000) mapped their HO-mutation in rapeseed and allocated it at linkage group 15 (LG15). These authors also concluded that a monogenic inheritance was found in their HO-rapeseed mutant which increase the oleic acid content by around 10% compared to the cultivar Wotan. Schierholt and Becker (2001) also notified that three or more minor genes have to be considered for accumulation of high amounts (>80%) of C18:1. In rapeseed as an amphidiploid species two *fad2* genes have been identified (Spiekermann, University of Hamburg, pers. comm., cited in

Möllers, 2002b). Current new *Brassica* mutants contain more than 85% C18:1 and 5% of the sum of C18:2+C18:3 (Auld et al., 1992, Schierholt et al., 2000).

The success of developing low erucic acid rapeseed was initiated by exploitation of the gene(s) affecting synthesis of erucic acid. Harvey and Downey (1964) reported that in the developing embryo of rapeseed, the amount of erucic acid relies on two genes which act in an additive manner. Introduction of recessive mutant alleles (e1 and e2) at each locus into the wild HEAR reduced erucic acid content from about 60% to 2% (Downey and Craig, 1964, Harvey and Downey, 1964). Within the genus *Brassica,* Downey (1987) stated that two loci are known to be linked with the synthesis of C22:1 in both amphidiploid species *B. napus* and *B. juncea*, while in the diploid species *B. rapa* there is only one locus. Furthermore, Ecke et al. (1995) mapped two loci controlling erucic acid synthesis on linkage groups 6 and 12 in a doubled haploid population of *B. napus*. Siebel and Pauls (1989) stated that at least five alleles contribute to the synthesis of erucic acid in *Brassica* as follows: e, E^a, E^b, E^c, and E^d with the synthesis of <1%, 10%, 15%, 30% and 3.5% erucic acid, respectively. Thus, by varying the allele composition present at the loci it is possible to modify erucic acid levels from essentially zero to over 50%. These two genes responsible for enhancement of erucic acid content in *Brassica napus* are homolog to the *fae1*-gene of *Arabidopsis thaliana* and other higher plants. Lassner et al. (1996) succeeded to insert the *fae1*-gene from *Simmondsia chinensis* under control of the seed specific napin promoter into rapeseed both low erucic acid rapeseed (LEAR) and HEAR types, and *Arabidopsis*. This *fae1*-gene increased C20:1+C22:1 by up to 20% in pooled T_2 seeds of the LEAR lines. Applying half seed methods in the T_3 the authors found a further increase up to 47% for the sum of those fatty acids. Increases of up to 45% of C20:1+C22:1 was found in pooled T_2 seeds of HEAR lines. In the interest of using the *fae1*-gene, Schröder-Pontoppidan et al. (1999) succeeded to transfer the *fae1*-gene from *Lesquerella fendleri* into *B. napus* cv. Hanna. After a series of back crosses

and selfings these authors found an increase of C22:1 synthesis from 16.5% in F_1 up to 61% in F_6 generation. Katavic et al. (2000) succeeded to insert the *fae1-gene* into HEAR cv. Hero using the napin promoter to improve erucic acid content to 51% whereas the wild type control has only 41%. Furthermore, this gene was also found to replace activity of the non functional gene in '00' quality cv. Westar. The transgenic Westar lines synthesised about 10-15% C20:1+C22:1.

However, in rapeseed oil C20:1 and C22:1 are found only at the first and third position of the triacylglycerol backbone. This is due to specificity of the *B. napus sn*-2-acyltransferase (lysophosphatidic acid acyltransferase; LPAAT) which excludes C20:1 and C22:1 from second triacylglycerol backbone position (Bernerth and Frentzen, 1990). Therefore, the theoretical threshold of the C22:1 content in rapeseed is naturally limited to 66%. Murphy (1995) suggested that insertion of 1-acylglycerol-3-phosphate acyltransferase (LPAAT) of *Limnanthes alba* could be used to esterify erucic acid to the second carbon position of the triacylglycerol backbone to enhance erucic acid formation in rapeseed. Attempts to elevate the C22:1 content through genetic modifications, focused on improving the capacity of the C22:1 synthesis (*fae1*-gene) as well as searching for LPAAT gene from different sources to enable the *sn-2* position to be filled with C22:1 (Table 1.2).

Lassner et al. (1995) inserted the LPAAT gene from *L. alba* into the HEAR oilseed rape cv. Reston. They reported that transgenic plants were able to insert C22:1 in the second backbone position in varying amounts from 12.4 to 22.3 mol%. In transgenic rapeseed line SVC144-2, the LPAAT from *L. douglasii* was also reported to enhance the formation of C22:1 at *sn-2* position by 28.3% and containing 32.1% C22:1 triacylglycerols (Brough et al., 1996). They also noted that in their transgenic material, the amount of erucic acid at the *sn*-2 position was correlated with the trierucin content.