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Erucic acid rapeseed: 1. Prospects of improvements

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Abstract – In the current context of boosting production of high erucic acid rapeseed, because of the wide range of its industrial applications, this literature review is designed to provide a general overview of available varieties, current knowledge of plant improvement and paths of developing research to increase competitiveness of varieties with high erucic acid content. A limited market dominated by a few companies, cropping burdens of high erucic acid rapeseed varieties among the majority "00" varieties and the still low erucic acid content in rapeseed, explains the reduced and uncompetitive varietal offers. To improve this situation, new varieties could be developed, thanks to the classical methods of selection and biotechnology.

Keywords: Erucic acid / rapeseed / variety / selection / biotechnology

Résumé – Colza érucique : partie 1. les pistes d'amélioration. Dans un contexte de relance de la production d'acide érucique, à partir du colza, en raison de ses nombreuses applications industrielles, ce dossier bibliographique a pour objectif de faire le point sur quelques variétés disponibles, les connaissances actuelles en matière d'amélioration des plantes et les pistes de recherche explorées pour améliorer la compétitivité des variétés éruciques. Un marché limité, dominé par quelques sociétés, les contraintes culturales des variétés riches en acide érucique parmi des variétés « 00 » majoritaires et la teneur encore limitée des graines de colza en acide érucique expliquent une offre variétale réduite et peu compétitive. Pour remédier à cette situation, de nouvelles variétés pourraient être développées, grâce aux techniques classiques de sélection et aux biotechnologies.

Mots clés : Acide érucique / colza / varieté / sélection / biotechnologie

1 Introduction

Several plant species in the Brassicaceae family produce erucic acid. The criterion for selecting plant species for erucic acid production are: enough agronomic productivity and a high enough seed erucic acid content. Among the Brassicaceae species the main ones are Crambe (*Crambe abyssinica*), the Ethiopian mustard (*Brassica carinata*) and oilseed rape (*Brassica napus*). Nevertheless the main interest in rapeseed is due to its higher agronomic productivity when compared with others species. Rapeseed oil "00" with low erucic acid and glucosinolates content has an average composition of 8% saturated fatty acids, 61% monounsaturated fatty acids and 9% polyunsaturated fatty acids. High erucic acid rapeseed (HEAR) contains around 50% of C22:1. For its technical use, it is desirable to increase the 22:1 content and to decrease the eicosenoic acid (20:1), the polyunsaturated fatty acid (PUFA, 18:2 + 18:3), and saturated fatty acid (16:0 + 18:0) content.

Erucic acid (*cis*-13-docosenoic acid, C22:1) is a long chain polyunsaturated fatty acid with 22 carbon atoms, with a double bound at the cis-13 position of the carbon chain. High erucic acid rapeseed (HEAR) oil is of interest for industrial purposes because erucic acid (22:1) and its derivatives are important renewable raw materials for the oleochemical industry. Its chemical formula is as follows:

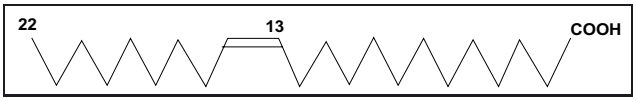

Fig. 1. Erucic acid chemical formula.

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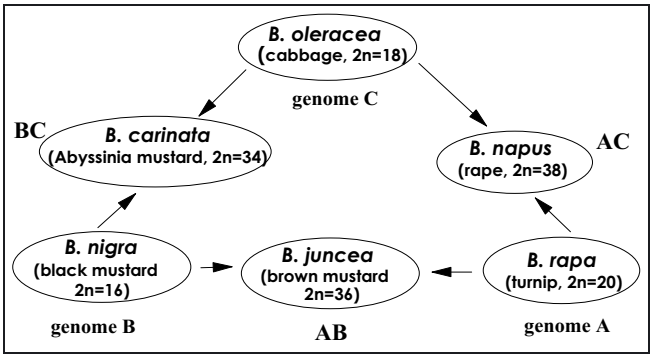

Fig. 2. Genetic relationships between several *Brassica* species.

Before the newer low erucic acid varieties were developed, the proportion of erucic acid was 45 to 50% of fatty acids in rapeseed oil.

Erucic acid is created by elongating oleoyl-CoA (adding two carbon units to the carbon chain) with malonyl-coA. The enzyme acyl-CoA elongase, which is responsible for erucic acid synthesis, is located in the endoplasmic reticulum. The elongation process studied in *Lunaria annua* consists of the following steps:

- oleoyl-CoA and malonyl-CoA condensation and creation of 3-ketoacyl-CoA;
- 3-ketoacyl CoA reduction to 3-hydroxyacyl-CoA;
- 3-hydroxyacyl-CoA dehydration and 2,3 transénoyl-CoA reduction.

The authors here present the first paper which deals with the prospects of improvements in erucic acid rapeseed. A second paper on variety supply, agronomic management and quality will then be presented in the OCL volume.

Improving HEAR rapeseed varieties is a difficult task which has to integrate precise knowledge of plant and seed metabolism and combine several approaches and technologies. Among the possibilities for plant breeders, two main areas are being explored in order to produce rapeseed with very high erucic acid content: the conventional selection methods, and biotechnology.

2 Conventional selection methods

A specific feature of *Brassica napus* is to have two genomes to form an amphidiploid species. Figure 2 shows the relationships between the genomes of the different *Brassica* species. This specific feature provideses opportunities as well as complexities for breeders. Resynthetized amphidiploids from *B. rapa* and *B. oleracea* is a possible strategy. Nevertheless, classical quantitative genetics has to deal with the presence of two closely related copies of a given gene for each trait, one on the A genome and the other on the C genome.

2.1 Comprehension of genetic determinism

When an interesting character is identified, it takes years to transfer it into the chosen material. During the last few years, marker assisted selection (RFLP probe, RAPD and microsatellites) has played a significant role in effective plant selection (Friedt and Lühs, 1995; Paterson *et al.*, 1991; Szewc-Mc Fadden *et al.*, 1996; Quiros *et al.*, 1994; Wang *et al.*, 1994; Waugh and Powell, 1992).

The two genes controlling erucic acid synthesis in rapeseed have been mapped on hybrid groups which are different from RFLP markers on doubled haploid (DH) lines derived by crossing "Mansholts Hamburger Raps" X "Samourai" (Friedt and Lühs, 1995; Uzunova *et al.*, 1995). In the same population, three quantitative trait loci (QTL) for seed oil content have been mapped, two of which mapped to the positions of the erucic acid genes (Uzunova *et al.*, 1995). Zhao *et al.* (2008) identified eight QTL for erucic acid content in the segregating doubled haploid (DH) population derived from a cross between two high erucic acid rapeseed cultivars. One QTL co-located with one of the two erucic acid genes (*FAE1*) and the remaining seven QTL were not related to the *FAE1* gene, but contributed to the 22:1 content.

Other genetic studies have revealed that erucic acid synthesis is controlled by two major gene loci *E1* (*Bn-FAE1.1*) and *E2* (*Bn-FAE1.2*), which have additive effects (Harvey and Downey, 1964; Lühs *et al.*, 1999; Stefansson, 1983). The two genes *Bn-FAE1.1* and *Bn-FAE1.2* mapped in *B. napus* representing the parental species *B. rapa* (A-genome) and *B. oleracea* (C-genome) *Fatty Acid Elongase* (*FAE1*) genes, showed polymorphisms (Fourmann *et al,.* 1998; Nath, 2008). Jönsson (1977), and Pourdad and Sachan (2003) reported that in rapeseed (*B. napus*), 22:1 content is controlled by alleles at one, one or two and two loci leading to 5–10%, 10–35% and more than 35% of 22:1, respectively. The two elongation steps from oleoyl-CoA to 22:1 are each controlled by alleles of two loci (Harvey and Downey, 1964; Stefansson, 1983). Ecke *et al.* (1995) and Jourdren *et al.* (1996) mapped the two loci determining erucic acid content in rapeseed populations using both random fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers. Assignment of the two loci to independent linkage groups was confirmed by a QTL approach by Thormann *et al.* (1996). However, the two loci do not contribute equally to erucic acid content. Each locus can have multiple alleles and leads to erucic acid contents ranging from 0.1% to about 60%. At least five alleles govern the erucic acid proportions in *Brassica,* including; *e*, *Ea*, *Eb*, *Ec* and *Ed*. Therefore, levels of erucic acid can be fixed at a large number of values ranging from < 1% to > 60% (Jönsson, 1977).

In winter rapeseed varieties, a single allele contributes to the production of approximately 16 to 17% C22:1 (Lühs and Friedt, 1994, 1995, 1997). Erucic acid biosynthesis involves elongation of oleic acid to eicosenoic acid and then to erucic acid. The elongation is the result of two cycles of a four-step process, in which 18:1-CoA and 20:1-CoA are used as substrates. In rapeseed, erucic acid biosynthesisis is controlled by the expression and the specificity of ß-ketoacyl-CoA synthase (KCS) (the enzyme responsible for the fatty acid elongation of oleic acid (18:1) to eicosenoic acid (20:1) and then to erucic acid), which is a gene similar to the *FAE1* (fatty acid elongase) gene present in *Arabidopsis thaliana* (James *et al.*, 1995; Lühs and Friedt, 1997). It is believed that the initial reaction of the four step elongation process is the rate-limiting step in the biosynthesis of erucic acid (Cassagne *et al.*, 1994). Crossing

of conventional HEAR to rapeseed with reduced contents of linoleic acid (18:2) and linolenic acid (18:3) did result in recombinant high erucic low polyunsaturated fatty acid (HELP) F3-plants which, however, did not show an increased 22:1 content compared to the parental HEAR genotype. This indicated that the β-ketoacyl-CoA synthase (KCS; *fae1* gene) activity may be limiting. The *fae1* gene encoding the KCS enzyme, has been cloned from a range of plant species and has been overexpressed under the control of a seed specific promoter in HEAR. However, only very minor increases in 22:1 content were reported (Han *et al*., 2001; Katavic *et al*., 2001). Even in combination with the expression of the *Ld-LPAAT* gene from *Limnanthes douglasii,* no substantial increase in the 22:1 content has been found (Han *et al.,* 2001).

Other bottlenecks in the pathway could be the availability of oleic acid for elongation to eicosenoic and erucic acids. In order to determine whether the availability of oleic acid can be a limiting factor, Sasongko and Möllers (2005) crossed HEAR (cv. Maplus) with high oleic acid rapeseed (HOAR) (Schierholt *et al.,* 2001) to recombine the genes for high 22:1 with those for high 18:1 (*i.e*. low content of polyunsaturated fatty acids). However, the recombinant line HELP did not show a significant change in 22:1 acid content, indicating that in this material the ß-ketoacyl-CoA synthase (KCS) activity may be limiting.

Nath (2008) showed a large variation in erucic acid content ranging from 44 to 72% with a mean of 58.8% in the F_2 plants (F_3 seeds) and from 50% to 72% with the mean of 64.8% in the F_3 plants (F_4 seeds) obtained by crossing transgenic HEAR winter rapeseed line (361.2B) and non-transgenic high erucic and low polyunsaturated winter rapeseed line 6575-1 HELP to produce F_1 plants which were selfed to produce F_1, F_2 and F_3 plants. The mean for 22:1 exhibited in the F3- population (F4-seeds) was 6% more compared to the mean of F2-population, indicating a response to selection. This compares favourably with the 63.2% of the transgenic parent 361.2B and 49.6% of the non-transgenic 6575-1 HELP (High Erucic and Low Polyunsaturated fatty acid) parents. The best F3-lines had a PUFA content of only 5 to 6%, which is about 10% lower than the PUFA content of the parent 361.2B. Results from regression analysis of the F2-population indicated that reduction in PUFA content by 10% led to a 6.3% increase in erucic acid content. The 72% erucic acid content achieved in the study marks a major breakthrough in breeding high erucic acid rapeseed. The trierucin content varied between 0 and 24.5% with a mean of 12.6%, respectively. In the segregating F_2 population, there were two groups; one group which lacked trierucin because it lacked *Limnanthes douglasii* lysophosphatidic acid acyltransferase (*Ld-LPAAT*) gene in the individuals of the F2 population. The group with trierucin showed a continuous variation as one would expect for a polygenic trait. Erucic acid content varied from 45% to 57% for the zero trierucin plants and in the trierucin group it varied from 48% to 72% in the segregating F2 population. Path coefficient analysis to partition the correlations of the different factors into direct and indirect effects showed little direct effect of trierucin content on erucic acid (0.06) content. Strong negative direct effects of oleic acid and PUFA content on erucic acid content (–0.58 and –0.73) were observed. Trierucin content also showed larger positive indirect effect *via* 18:1, PUFA and 20:1 content on erucic acid than direct effect. Eicosenoic acid showed indirect negative effect *via* oleic acid as much as direct effect on erucic acid content (Nath, 2008). This information and material will be valuable for future approaches to increase erucic acid content in rapeseed beyond the levels currently obtained. Nath (2008) also showed that the erucic acid content varied from 35–59% in the segregating DH-lines, 49–52% in 6575-1 HELP parental lines and, 46–48% in transgenic resynthesised high 22:1 rapeseed line TNKAT. TNKAT, a resynthesised transgenic winter rapeseed line (RS306) was derived from an interspecific cross between Yellow Sarson (*Brassica rapa*) for A-genome (*FAE1.1*) and cauliflower (*Brassica oleracea* sp. *capitata*) cv. Super Regama for C-genome (*fae1.2*) as outlined by Lühs and Friedt (1994). RS306 was used for *Agrobacterium* mediated transformation to produce the TNKAT line carrying a single transgene copy (chimeric *Bn-FAE1.1* with *Ld-LPAAT* where both were under the control of the seed specific napin promoter (Han *et al*., 2001).

Previous work on a *B. juncea* RFLP map (Cheung *et al.*, 1998) identified two QTL on linkage groups 7 and 4 which explained approximately 85% of the phenotypic variance of C22:1. A candidate-gene approach has helped locate two genes encoding the elongase enzyme within the confidence interval of the two QTLs. These results are like those obtained by a similar approach on another amphidiploid obtained by integrating the genomes of *B. rapa* and *B. napus* (Ecke *et al.*, 1995; Jourden *et al*., 1996) using common markers, thus suggesting that the elongase genes are present in the genome of *B. rapa*.

2.2 Use of doubled haploid lines

The use of classical methods for rapeseed breeding (genealogical selection) to change the fatty acid composition would take at least 10–12 years. A faster method would include the application of microspore culture techniques to produce DH populations (Friedt and Lühs, 1995; Kontowski and Friedt, 1994; Lühs and Friedt, 1994, 1995; Nath *et al*., 2007; Thierfelder *et al*., 1993). Since the development of the isolated microspore culture technique for the production of homozygous DH lines in oilseed rape (*Brassica napus* L.) by Lichter (1982), this method has gained considerable importance in rapeseed breeding programmes. The method has been optimised variously (Chen *et al.*, 1994; Iqbal *et al.,* 1994; Möllers *et al.,* 1994; Zaki and Dickinson, 1991).

Crosses were made between cell lines derived from cultivars of winter rapeseed relatively rich in erucic acid. Parental and F1 progeny lines were used as donors for microspore culture and as a source of microspore derived embryos (MDEs) which are cultured to produce haploid plants. The haploid plants were vernalized and treated with colchicine to double the chromosomes to produce DH lines. This method provided varieties with high levels of erucic acid (up to 60%) (Friedt and Lühs, 1995; Jourdren *et al*., 1996). Furthermore, Nath *et al.* (2007) developed a simple protocol for simultaneously extracting lipids (for oil quality analysis) and DNA (for marker-assisted selection) from single cotyledons dissected from MDEs of *Brassica napus*, suitable for fatty acid analysis, PCR amplification and regeneration of the rest of the embryo,

allowing selection of valuable MDE genotypes at an early stage in segregating MDE populations; derived by crossing the homozygous transgenic resynthesised high erucic acid rapeseed line RS306, carrying a single T-DNA with two chimeric genes (Han *et al*., 2001), with the high erucic acid winter rapeseed line 6575-1 (Sasongko and Möllers, 2005) segregating for two linked transgenes, *fae1* and *plsC*, affecting the fatty acid composition. The early identification of 50% MDE individual plants carrying the desired transgenes, along with high expression of the trait, allows for early selection for plantlet regeneration, and reduces the probability of the inclusion of undesirable genotypes and exclusion of desirable recombinant genotypes among the total number of regenerated MDEs. Hence, marker-assisted selection (MAS) at the *in vitro* stage would screen a larger population of MDEs and reduce greenhouse costs. Unequivocal selection for absence of, intermediate and high erucic acid (Albrecht *et al.,* 1995) and oleic acid (Möllers *et al.,* 2000) was possible in segregating MDE populations by determining the fatty acid composition by gas liquid chromatography (GLC) from single cotyledons dissected from MDEs. However, the application of this early *in vitro* selection system is limited to those traits that can be rapidly and cost effectively analysed and for which a close correlation between the MDEs and the seeds from the regenerated plants has been shown. Furthermore, phenotypic results may be confounded by MDE genotype x environment interactions, *i.e.*, *in vitro* culture conditions may differently affect the fatty acid composition of storage lipids in individual MDE genotypes. Such shortcomings are overcome if MAS is applied concomitantly. The erucic acid content ranged from 14.1 to 42.7% in the MDE genotypes where both *plsC* (from *Limnanthes douglasii*, accession no: X83266) and *fae1* (accession no : AF274759) genes were expressed and 14.7 to 30% in the MDE genotypes where only *plsC* was expressed. However, results from fatty acid analysis did not reveal a pronounced effect of the transgene on the erucic acid content of the MDE and were not correlated with the erucic acid contents of the seeds obtained from the corresponding DH plants in the green house. The mean erucic content of 54 DH lines with the transgene was 2.3% lower than the 36 DH lines lacking the transgene. A strong negative correlation was found between erucic acid and polyunsaturated fatty acid content (18:2 + 18:3; $rs = -0.40^{**}$). This result indicates that the ectopic *FAE1.1* gene may not be functional (Nath, 2008). Futhermore, it was observed that dividing the DH population into half according to their PUFA (polyunsaturated fatty acid) content revealed that a 8.1% reduction in PUFA content (mean = 11.4%) in the DH lines resulted in an increase in erucic acid content by 3.7% when compared to the DH lines with high PUFA content (mean = 19.5%). The best DH line (IV-10-F-6) had 59.1% erucic acid in the seed oil. This was 9% more than the higher erucic acid parent 6575-1 HELP (Nath, 2008). Although the best three DH lines selected for high 22:1 were transgenic, it seems that transgene (*fae1*) cannot increase the 22:1 content but interacting with other factors like low PUFA genes play a vital role in increasing 22:1 proportions by reducing PUFA content.

The fatty acid composition of the MDE and the mature seeds being substantially identical, the immature embryos were used to study the composition of the storage compounds (lipids, proteins) and more specifically, the biosynthesis of triacylglycerols (TAG) containing high erucic acid proportions. Because of their high enzymatic activity, homogenates of immature embryos are able to make TAG. It has been demonstrated that they can synthetize trierucin in the presence of 1,2-dierucylglycerol and erucyl-CoA (Taylor *et al*., 1990, 1991, 1992; Friedt and Lühs, 1995). The technique of the immature embryos can be used as a method of early sorting, coupled with the one of DHs. Among the early sorting techniques, it is also possible to make measurements on the half-cotyledons.

The range of the erucic acid content recorded by Nath (2008) in 90 DH lines derived from the cross between TNKAT × 6575-1 HELP was 34.6 to 59.1% (mean = 47.1) and the heritability was 0.88 whereas the oil content range was 28.3 to 50.7% (mean = 40.5) and the heritability of oil content was 0.82, respectively. The oil and erucic acid content of the parental line TNKAT were 40.8% and 46.1% and that of the parental line 6575-1 were 46.4% and 50.4%, respectively.

3 Applications of biotechnology

3.1 New synthesis of Brassica napus from B. rapa and B. oleracea

Brassica napus is a natural amphidiploid species, resulting from the spontaneous hybridization of *B. rapa* (A-genome) and *B. oleracea* (C-genome). But it turns out that the genetic variability of *B. napus* is lesser than that of *B. rapa* and *B. oleracea*, which are highly polymorphic. This wide variability of the parents can be exploited by manipulation and re-synthesis of *B. napus* from the original parents. The new variety of *B. napus* obtained is genetically intermediate between the two parents, but very different from the natural forms of *B. napus* (Becker *et al*., 1995; Chen and Heneen, 1989; Engqvist and Becker, 1994; Friedt and Lühs, 1995; Kräling, 1987; Lydiate *et al*., 1993; Olsson, 1986; Song *et al*., 1993; Thierfelder *et al*., 1993).

Currently, erucic acid content in *B. napus* is limited to 55%, the average content ranging mostly between 45 and 50% (Friedt and Lühs, 1995; Malher and Auld, 1988; Lühs and Friedt, 1994, 1995). However, the variation range of erucic acid in both parents is greater, ranging from 30.1 to 61.4% in *B. rapa* and from 28.2 to 63.4% in *B. oleracea*. Biotechnology helped in creating a new amphidiploid from parents rich in erucic acid (about 55–60%). New somatic hybrid rapeseed was created by the fusion of protoplasts of *Brassica oleracea var botrytis* and *Brassica rapa var oleifera*, which were selected for the high erucic acid content in their seed oil. One of the regenerated plants contained 57.4% erucic acid. The fatty acid composition of plants in the R1 generation was stable and was coupled with increasing female fertility (Heath and Earle, 1995).

Genetic studies on the newly synthesized material showed that the genes responsible for erucic acid synthesis had an allelic contribution ranging from 16 to 17% relative to the total content of erucic acid. However, using this technique, limits the synthesis of trierucin (Lühs and Friedt, 1994, 1995).

Some cauliflowers genotypes were able to esterify the erucic acid at the *sn*-2-position of the triacylglycerols (Taylor *et al*., 1994; Friedt et Lühs, 1995). However, trierucin was not found in the seed oil in a mutant form of *B. oleracea*. According to Downey and Taylor (1996), previous work by the same team (Taylor *et al*., 1995) produced cauliflowers with 60% erucic acid in their seeds, including 20–25% in the *sn*-2-position. *B. oleracea* × *B. rapa* and *B. napus* × *B. oleracea* crosses are expected to result in high erucic acid varieties with low glucosinolate content and about 75% C22:1, respectively.

Another promising method for introducing the desired traits is the fusion of protoplasts of plants of the crucifer family with wild and cultivated varieties of *B. napus* or *B. juncea*. Thus, fertile hybrids between *B. napus* and *Arabidopsis thaliana* (Forsberg *et al*., 1994; Friedt and Lühs, 1995) or between *B. napus* and *Thlaspi perfoliatum* (Fahleson *et al*., 1994; Friedt and Lühs, 1995) were obtained. However, this method is limited by several factors: the problem of sustainability of plants, fewer fertile plants, new obtained material far from being cultivable (and therefore requiring series of backcrosses, *i.e*. crossing back the interspecific hybrid with the cultivated species, in order to regain its traits, along with selection to retain the advantageous traits).

3.2 Trierucin synthesis (trierucylglycerol)

Although the main component of triacylglycerol in rapeseed is erucic acid, the long fatty acid chain can be attached to the *sn*-1 and *sn*-3-positions of glycerol, but is excluded from the *sn*-2-position. Therefore, we do not find any trierucin in rapeseed oil and the theoretical limit of the erucic acid content in rapeseed is 66%. Several studies suggested that two biochemical steps are critical for improvement of erucic acid production in rapeseed: membrane bound fatty-acid elongation and lysophosphatidic acid acyltransferase (LPAAT) activity leading to the biosynthesis of trierucin (Bernerth and Frentzen, 1990; Creach *et al*., 1993; Friedt and Lühs, 1995; Taylor *et al*., 1993).

Thus, the two preferential targets for genetic research are: acyl-CoA elongase and lysophosphatidic acyltransferase.

3.2.1 The lysophospatidic acid acyltransferase (LPAAT)

In recent years, efforts related to cloning acyltransferases genes focused on erucyl-CoA preferring acyltransferase (LPAAT) were made. The first gene of LPAAT, *plsC* from *E. coli*, was cloned from a complementary homologous DNA, of a mutant strain of *E. coli*, which was deficient in the activity of LPA-AT (Coleman, 1990, 1992; Frentzen, 1998). The gene of an erucoyl-CoA preferring *sn*-2 acyltransferase from *Limnanthes douglasii* (*Ld-LPAAT*) has been successfully cloned and overexpressed in rapeseed (Brough *et al.,* 1996; Brown *et al.,* 1995; Friedt and Lühs, 1998; Hanke *et al.,* 1995; Lassner *et al*., 1995; Frentzen, 1998). However, the overall proportions of 22:1 in the seed oil did not increase. Zou *et al*. (1997) have confirmed that the yeast (*Saccharomyces cerevisiae*) *SLC1-1* gene encodes *sn*-2-acyltransferase capable of acylating *sn*-1-oleoyl-lysophosphatidic acid using a range of acyl-CoA thioesters, including 22:1-CoA. However, neither the meadowfoam nor the yeast-*LPAAT* transgene approach was successful in achieving high trierucin content in HEAR *B. napus* seed oil. The failure to significantly increase the 22:1 level by engineering *LPAAT* could be due to a limitation in the acyl-CoA pool in the cytosol, which is required to support high levels of trierucin synthesis (Lühs *et al*., 1999; Sasongko and Möllers, 2005). This hypothesis was supported by an increase in the levels of 22:1 (48 to 53%) in transgenic "Hero" plants which expressed the yeast *fae1* when compared with 43% of 22:1 in the wild-type control lines (Katavic *et al.,* 2000). Similar results were obtained with the expression of *Arabidopsis* and *B. napus fae1* in rapeseed (Han *et al.,* 2001; Katavic *et al.,* 2001; Wilmer *et al.,* 2003). Thus, these studies show that the proportions of 22:1 in rapeseed oil is limited by both 22:1 synthesis and its subsequent incorporation into TAG (Katavic *et al*., 2000). HEAR oil could eventually be produced by combining these and other genetic modifications. Weier *et al*. (1997) suggested that the level of trierucin depends not only on the activity of the introduced *sn*-2-acyltransferase but also on other biosynthesis or incorporation steps. It is possible that the levels of erucoyl-CoA in the seed acyl-CoA pool may be too low to allow high levels of trierucin biosynthesis. If this is the case, then overexpression of genes regulating very long chain fatty acids (VLCFAs) biosynthesis may be required to boost very long-chain acyl-CoA availability for incorporation into seed triacylglycerols (TAGs). Analysis of the cDNA encoding the microsomal LPAAT revealed two distinct classes of genes, A and B (Frentzen, 1998; James *et al*., 1995). The class A microsomal LPAATs defined by Frentzen (1998) possess substrate preferences for C18:1-CoA typical of enzymes involved in membrane lipid synthesis and are ubiquitously expressed in the plant. In contrast, individual members of the class B LPAATs display preferences for distinct, unusual saturated or unsaturated acyl groups and are normally expressed in storage organs. Although class B LPAATs have been exploited to alter the stereochemical composition of rapeseed (*Brassica napus*) oil to permit the incorporation of modified fatty acids at *sn*-2 (Knutzon *et al.*, 1999; Lassner *et al.*, 1995), a significant increase in the total amount of unusual fatty acid was not accomplished by the expression of the class B LPAATs alone. In contrast, the transformation of rapeseed and *Arabidopsis thaliana* with a yeast gene encoding a variant LPAAT, *SLC1-1*, capable of accepting very long chain fatty acyl (VLCFA)-CoA substrates resulted in an increase in the total VLCFAs and, unexpectedly, in total oil content (Zou *et al.,* 1997).

In a study by Nath (2008), seed oil from the non-transgenic parent (6575-1 HELP) predominantly contained oleic acid at the *sn*-2-position (73.3%), while very long chain fatty acid (22:1) was detectable in trace amounts only. On the other hand, the oil from the transgenic parents (TNKAT and 361.2B) and the selected best DH and F3 lines (F4-seeds) contained higher amounts of 22:1 and correspondingly lower proportions of 18:1, respectively. The highest amount of 22:1 at the *sn*-2-position was found in the F3 line (III-G-7), followed by the DH line (IV-10-F-6) with values of 65.3% and 40.3%, respectively. These *sn*-2 compositions of the transgenic seed oils correlated with the 22:1-CoA specificity of the expression of the *Ld-LPAAT* gene from *L. douglasii.* This result is

in agreement with the observation of Weier *et al.* (1997), and Han *et al*. (2001). Hence, lipid analyses revealed that the introduced *Ld-LPAAT* gene effectively competes with the endogenous rapeseed enzyme and preferentially incorporates 22:1 into the *sn*-2-position of the glycerol backbone. However, considerable amounts of oleic acid were also detected at the *sn*-2-position, indicating that endogenous *Bn-LPAAT* activity may be limiting for achieving higher erucic acid content at the *sn*-2 of *Ld-LPAAT* overexpressing rapeseed lines.

Molecular biologists are trying to regulate the biosynthesis of trierucin by replacing rapeseed LPAAT with corresponding enzymes known for its affinity for erucyl-CoA as substrate from appropriate donors like *Limnanthes douglasii* and *Limnanthes alba* (Friedt and Lühs, 1995; Taylor *et al*. 1993; Wolter *et al*., 1991, 1995). *Limnanthes* seed oil consists of more than 90% of VLCFAs (Lassner *et al*., 1995; Miller *et al*., 1964). The *sn*-2-position of *Limnanthes douglasii*, has a higher content of C22:1 relative to the total oil composition (Lassner *et al*., 1995; Phillips *et al.*, 1971). Thus, cDNA encoding 1-acylglycerol-3-phosphate acyltransferase in *L. douglasii* was introduced into rape. Trierucin was absent in the control plants and was found at a level of 0.4% and 2.8% in two transgenic plants. The fatty acid analysis did not find erucic acid in the *sn*-2-position in the control plants, but the two transgenic plants had 9% and 28.3% of erucic acid in the *sn*-2-position. These results showed that the gene encoding 1-acylglycerol-3-phosphate acyltransferase in *L. douglasii* may be functional in rapeseed and allow the incorporation of erucic acid at the *sn*-2-position of the triacylglycerols in rapeseed (Brough *et al*., 1996).

The same strategy has been used by Calgene (Lassner *et al*., 1995). The cDNA encoding LPAAT had been isolated from seeds of *Limnanthes alba* and was expressed in the seeds of transgenic rapeseed. Erucic acid was seen in the *sn*-2-position of the glycerols in the transgenic plants but was not seen in the control plants. The *LPAAT* gene obtained from *L. alba* incorporated erucic acid in the *sn*-2-position of the rapeseed triglyceride. The highest level of erucic acid in the *sn*-2-position observed in the oil of the transgenic plants is 22.3%. However, these manipulations do not significantly increase the total concentration of erucic acid content which was 40% in the control plants, and a little over 50% in transgenic plants. Recently, the genes encoding the elongase enzyme have been isolated and used to increase the erucic acid content of rapeseed oil (James *et al*., 1995; Lassner *et al*., 1996). The expression of these genes, in addition to the use of LPAAT from *Limnanthes*, should allow a sharp increase in levels of erucic acid in the rapeseed cultivars.

The *LPAAT* gene in *E. coli* has a broad specificity for acyl-CoA and may use VLCFAs. The oil in the transformed plants (Erox) with the gene from *L. douglasii* contains about 40% of C22:1, but less than 1% of C20:1 in the *sn*-2-position, whereas the transformed plants (Erox) with the *E. coli* gene have ten times less C22:1 and ten times more C20:1 in the *sn*-2-position (Weier *et al*., 1998). These results suggest a kind of specificity or preference of *E. coli* acyltransferase for C20:1 with respect to C 22:1. The increase in the total amount of C22:1 was not much: 42% for the control and 33–50% for the different Erox transformants, the amounts of C20:1 + C22:1 ranging from 46.2 to 60.4% against 54.8 in the unmodified Erox control plant. The authors hope to increase the proportions of C22:1 at the *sn*-2-position at the expense of C20:1 by changing the elongase activities.

However, as it has already been pointed out that the high levels of erucic acid in the *sn*-2-position in the oil of transgenic plants containing the gene LPAAT from *Limnanthes* does not increase the total concentration of C22:1 (Brough *et al.*, 1996; Frentzen, 1998; Lassner *et al.*, 1995; Weier *et al.*, 1997). It is therefore likely that in the transformed erucic acid rich rapeseed, the total amount of C22:1 is limited predominantly by the enzyme activities for the production of C22:1-CoA, especially by the activity of β-ketoacyl-CoA synthase (Frentzen, 1998; Lassner *et al*., 1996; Millar and Kunst, 1997). Recently, this hypothesis has been confirmed by the development of transgenic erucic acid rich rapeseed, expressing both the LPA- AT from *L. alba* and β-ketoacyl-CoA synthase (Frentzen, 1998). The expression of the additional β-ketoacyl-CoA synthase has caused an increase in the total concentration of erucic acid by about 45–60% and an increase in trierucin content in the oil of transgenic plants (Tab. 7).

Finally, new perspectives have been provided by Zou's team (Frentzen, 1998; Zou *et al*., 1997). They developed transgenic rapeseed with high erucic acid content expressing a variant LPAAT yeast (*Saccharomyces cerevisiae*), encoded by the gene *SLC1-1* (Frentzen, 1998; Nagiec *et al*., 1993), which can use a wide range of acyl-CoA thioesters with long and very long chains and also seems to have low specificity for acyl acceptors (Frentzen, 1998; Zou *et al*., 1997). However, it appears that the expression of the LPAAT from yeast does not only affect the stereochemical composition of the oil of the transgenic plants, but also the proportions of C22:1 and oil. Thus, the oil content increases from 34% of the dry weight in the seeds of the control plants to 41% in some transgenic plants, and the concentration of erucic acid ranges from 45% to 56%, respectively. Furthermore, the erucic acid concentration in the *sn*-2-position of the transgenic plants is low and does not exceed 4%, respectively. Therefore, the increase in the content of C22:1 cannot be attributed only to the *sn*-2-position, but to the concomitant increase in the concentrations of erucic acid at the *sn*-1-position and *sn*-3-positions. These findings, as well as the increase in the oil content, highlight the effect of the yeast LPAAT which allows an increase in the flow of fatty acids in the triglycerides biosynthetic pathway. The use of acyl-CoA thioesters by all acyltransferases involved in the biosynthesis of triglycerides seems to stimulate the biosynthesis of VLCFAs probably by removing feedback inhibition (Frentzen, 1998; Zou *et al*., 1997). The results obtained with the yeast LPAAT should be attributed to the specific properties of this enzyme. Thus, the low specificity of acyl acceptors of yeast LPAAT might be the decisive property that would differentiate it from the *LPAAT* genes obtained from other species. This hypothesis needs to be confirmed by further research (Tab. 1).

Recently, Nath *et al.* (2009) achieved around 72% erucic acid content and also reported that further increases in erucic acid content can be expected from progress in reducing the contents of the remaining fatty acids, mainly oleic acid, polyunsaturated fatty acids and eicosenoic acid.

Table 1. Modified erucic acid lines expressing a foreign acyl-transferase gene allowing to bind an erucic acid in *sn*-2-position of triglycerol (Frentzen, 1998). *RS: resynthetized lines.

Genotype	C22-1 content	LPAAT gene source	% max of *sn*-2	Reference	Origin-support
	Control unmodified plants				
cv Reston	40%	*L. alba*	22%	Lassner *et al.*, 1995	US Calgène
line	32%	*L. douglasii*	32%	Brough *et al.*, 1996	UK. Nickerson
cv Erox	46%	*L. douglasii*	35%	Weier *et al.*, 1997	ALL NPZ, Agrevo
RS* lines	60%	*L. douglasii*	41%	Weier *et al.*, 1997	ALL NPZ, Agrevo
cv Erox	46%	*E. coli*	4%	Weier *et al.*, 1998	ALL NPZ, Agrevo
cv Hero	45%	*S. cerevisiae*	4%	Zou *et al.*, 1997	CAN Saskatoon

3.2.2 The elongase

If binding of erucic acid at the *sn*-2-position is required to exceed the theoretical threshold of 66% C22:1, it has been observed that the increase in erucic acid content also requires the optimization of the transformation of fatty acids into C16 to C18 or longer chain C20 or C22. The regulation stage of the enzymatic activity has been studied more than the biochemical or molecular aspects. This is partly due to the complexity of the enzyme system and its regulations. The structure and functioning of the acyl-CoA elongase complex is poorly understood because of the difficulty in purifying functional membrane proteins to homogeneity. The acyl-CoA elongase complex has been partially purified from developing rapeseed embryos and has resulted in the enrichment of four proteins between 54 and 67 kDa in size (Creach and Lessire, 1993). The β-ketoacyl-CoA synthase (KCS) was purified from jojoba embryos by Lassner *et al.* (1996). The corresponding cDNA, homologous to the *Arabidopsis Fatty Acid Elongation1* (*FAE1*) gene (James *et al.*, 1995), was used to transform rapeseed plants. Subsequent KCS activity in developing embryos of low erucic acid rapeseed (LEAR) plants resulted in an enrichment of VLCFAs (up to 33.5% by weight) in the seed oil, thereby demonstrating that KCS activity had been restored.

Additional transgenic approaches to increase erucic acid

(i) ATP-citrate lyase (ACL):

The acetyl-CoA pool required for new fatty acid biosynthesis is primarily generated by the plastidic isoform of the pyruvate dehydrogenase complex (Ke *et al.*, 2000). Fatty acid elongation is a cytosolic process, and cytosolic ATP-citrate lyase (ACL) generates the required acetyl-CoA precursor. The temporal distribution of ATP-citrate lyase (ACL) activity in developing seeds of rapeseed closely paralleled both that of acetyl-CoA carboxylase (ACCase) in the cytosol and the overall rate of lipid biosynthesis (Fatland *et al.*, 2002). In the cytosol, acetyl-CoA can be carboxylated by ACCase to form malonyl-CoA and hence is converted to long chain fatty acids. Therefore, increased activity of ATP-citrate lyase (ACL) gene will help to produce more acetyl-CoA in the cytosol from mitochondria, which might produce the necessary malonyl-CoA for long chain fatty acid biosynthesis.

(ii) Cytosolic acetyl-CoA carboxylase (ACCase):

In the cytosol, plant fatty acids are synthesized by the action of acetyl-CoA carboxylase (ACCase) which converts acetyl-CoA to malonyl-CoA. Cytosolic acetyl-CoA is metabolized via one of three mechanisms: carboxylation, condensation, or acetylation. In the cytosol, acetyl-CoA can be carboxylated by acetyl-CoA carboxylase to form malonyl-CoA (Fatland *et al.*, 2002). Cytosolic malonyl-CoA is required for the biosynthesis of long chain fatty acids like erucic acid (22:1). Therefore, overexpression of cytoplasmic acetyl-CoA carboxylase (ACCase) and ATP-citrate lyase in transgenic high erucic and low polyunsaturated fatty acid (HELP) rapeseed lines could help to further increase erucic acid content in the seed oil of rapeseed.

(iii) *Brassica napus* lysophosphatidic acid acyltransferase (*Bn-LPAAT*) antisense:

During glycerolipid synthesis, three distinct acyltransferases are responsible for the sequential transfer of acyl groups from acyl thioesters to the glycerol backbone forming TAG (Ohlrogge and Browse, 1995). Among them lysophosphatidic acid acyltransferase (LPAAT) catalyzes the second acylation reaction so that the central position (*sn*-2) in the biosynthesis of the various glycerolipids, is formed. Therefore, LPAAT substrate specificities are decisive for establishing the fatty acid pattern of TAG. In conventional rapeseed the microsomal LPAAT has a pronounced specificity for 18:1 over other fatty acids. Therefore, the microsomal pathway results in the formation of glycerolipids in which the *sn*-2-position is specifically esterified with oleic acid (Frentzen, 1998). The enzyme activity of the endogenous rapeseed LPAAT (*Bn-LPAAT*) competes with the activity of the erucoyl-CoA specific *Ld-LPAAT* from *Limnanthes douglasii*. Down-regulation of endogenous *Bn-LPAAT* gene by antisense technique or mutation could help to increase 22:1 content at *sn*-2-position as well as in the seed oil (Nath, 2008).

Work to purify and characterize the enzyme complexes, acting in response to the carbon chain elongation, are being carried out under a French project supported by CETIOM and ONIDOL and funded by MENESR. Rustica and Serasem are also participating in these projects. The different research teams are INRA Versailles and INRA Le Rheu, University of

Bordeaux 2, Laboratory of Plant Physiology and Molecular Biology of University of Perpignan, University Pierre and Marie Curie of Paris. The Bordeaux team characterized the acyl-CoA elongase as a multi-membranes enzyme complex including at least two elongases, one responsible for the formation of C20:1 and the other C22:1 (Creach *et al*., 1993). The Laboratory of Plant Physiology and Molecular Biology of Perpignan (Roscoe and Delseny, 1997) works on the isolation of genes encoding the elongase which are necessary to assemble of triacylglycerols enzymes. Studies have characterized the structure and expression of the gene *FAE1* (elongase) rape. In collaboration with INRA Rennes, they showed that the two cDNA homologous to *FAE1* from immature embryos were linked to the E1 locus associated with the variation in erucic acid content in rapeseed (Jourden *et al*., 1996). A series of truncated *FAE1* sequences were used to express portions of the *FAE1* protein in *E. coli* for functional studies and to isolate cDNA encoding the sub-units of the elongase complex.

Ongoing studies on elongase showed more complex issues than the ones initially planned, because of its different forms and features as a membrane enzyme. The results of this work is in progress and still confidential.

4 Coming varieties and conclusion

Progress in improving agronomic traits and erucic acid content in oil will provide the rapeseed varieties rich in erucic acid:

- Higher yields than the erucic rapeseed varieties currently available: close to those of conventional rapeseed yields, against the current yields which are lower by 15%, respectively.
- Lower glucosinolate content, with a generalization of varieties +0;
- Richer erucic acid content (50–55%), pending very high erucic acid content varieties (80% minimum), obtained by genetic modification that might appear on the market in the medium to long term (Anonymous, 1998).

However, the successful production of erucic acid from rapeseed will depend on its competitiveness, when compared with products derived from other fatty acids (oleic and stearic) and also in the interest in rapeseed, when compared to other crucifers such as mustard, turnip, and crambe. Through the pursuit of agricultural work to improve yield, *Crambe abyssinica* seems to be the most competitive alternative plant (Merrien, 1997).

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