DEVELOPMENT IN THE BREEDING OF RAPESEED OIL FOR INDUSTRIAL PURPOSES

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ABSTRACT

During the last decade one of the most important objectives in rapeseed breeding has been the genetic modification of seed oil by changing the proportion of fatty acids in order to obtain tailor-made raw materials suitable for industrial purposes. Since oilseed rape (*Brassica napus*) is one of the few economically interesting species most amenable to biotechnology, a couple of different breeding procedures, i.e., generation of doubled-haploid lines, interspecific hybridization, molecular marker techniques and genetic engineering through recombinant DNA technology, are involved in developing improved basic stocks and cultivars possessing the novel desirable trait. This review describes the genetic modifications of fatty acid chain length - both longer and shorter than the common C16 to C18 range - that have been recently achieved in oilseed rape.

INTRODUCTION

In the past few decades rapeseed - derived from several locally distributed *Brassica* spp. - has become the world's third most important source of vegetable oil. According to the long-term analysis *Oil World 2012* forecasting the next 20 years, rapeseed oil will maintain its position accounting for about 14% of the world vegetable oil production. The primary demand is made up by food and animal feed industry supplied by 'double-low' or 'canola' quality (Anonymous, 1994; Lühs and Friedt, 1994a). With regard to production of renewable resources on set-aside land (e.g., in Germany in 1994 some 142,000 ha industrial rapeseed), the opportunity has grown to produce Biodiesel feedstocks or speciality oils for several industrial niche markets. In particular, high erucic acid oils as a typical industrial feedstock are of growing interest (e.g., in 1994 approx. 8,000 ha HEAR in Germany on set-aside land). However, oils and fats now available to the industry are derived in large part from those usually used for food purposes. But these are not always the best starting materials for the production of oleochemicals.

From an industrial point of view, the usability of a seed oil is targeted towards only one of its constituent fatty acids. A maximum content of the desired fatty acid will not only decrease the amount of waste, but it can result in considerable savings in downstream processing costs, too. The majority of fatty acids used for industrial purposes consists of unbranched hydrocarbon chains with a range from 16 to 18 carbon atoms. They are further transformed by chemical reactions involving either the carboxyl group or the hydrocarbon chain. On a commercial scale nearly all, i.e., 96%

of these reactions are directed to the derivatisation of the carboxyl group, only 4% deal with the modification of the hydrocarbon side chain. However, increased flexibility and new derivatives have to come from oils providing fatty acids with unusual chain lengths and/or functionalities, such as unique double bond positions or functional groups, e.g., hydroxy, epoxy, acetylenic, or keto groups (cf. Lühs and Friedt, 1994b).
Evidently, some of these radical changes in fatty acid composition will not be realized satisfactorally by conventional methods of plant breeding - even not by assistance of biotechnology. In these cases sophisticated molecular methods are required for transferring specific foreign genes between distant species. For instance, it is still the most prominent subject of investigation in several laboratories to create a temperate, annual crop like oilseed rape as a commercial source of high contents of lauric acid or trierucin. Promising examples of modification of fatty acid and storage lipid metabolism, which can be anticipated for the nearer future, are reported in detail elsewhere (Kridl et al., 1993; Murphy, 1994a; Ohlrogge, 1994; Knauf, 1994). More information on overall aspects of "designing" new oil crops has been compiled by Murphy (1994b).

TARGET 1: RAPESEED OIL WITH MAXIMUM ERUCIC ACID CONTENT

Markets of C22 oleochemicals

Vegetable oils rich in erucic acid can be regarded as a typical industrial feedstock directed to a highly specialized, but growing market. Today, C22 oleochemicals - derived from erucic acid (*cis*-13-docosenoic acid, C22:1) and its hydrogenation product behenic acid (C22:0) - have a wide range of applications due to their excellent physicochemical characters such as clinginess to surfaces, emolliency, hydrophobicity and lubricity. Actually, most of the oil is processed into erucamide, which is used as an antiblock, slip-promoting additive in the production of polyolefine films. For this special application the properties of the erucic acid derivative are highly valued, even though it costs roughly twice as much as oleamide. The list of further C22-based products includes food additives, cosmetics and personal care products (e.g., for hair care), surfactants and detergents (e.g., textile softening), photographic and recording materials, plastics and plastic additives, lubricants and fuel supplements (e.g., pour point depressants), and a lot of other uses. Successful commercialisation of C22-based products depends on feedstock material costs and co-product credits, the two most important factors in the economic feasibility. Although the slightly better efficiency and usefulness of higher chain length materials is well recognized, they will not be exploited at present, because there are a number of alternative products, derived from oleic and stearic acid, with which C22-based products have to compete on the basis of price (Sonntag, 1991; Lühs and Friedt, 1994b; Leonard, 1994).

Commercial sources of erucic acid

With the advent of low-erucic acid rapeseed cultivars, the production of traditional high-erucic acid rapeseed (HEAR) has waned rapidly worldwide (Lühs and Friedt, 1994a). Consequently, during the past 20 years the development of alternative crops for production of erucic acid has been strongly promoted. Among potential

candidates in the *Cruciferae* family, crambe (*Crambe abyssinica*) as a new annual crop has received most attention in this regard (Lühs and Friedt, 1994b; Van Soest, 1994). For example, in the United States some 25,000 hectares of crambe cropland were contracted by industry in 1993 for the production of high-erucic oils (Anonymous, 1993). In Europe, breeding research and experimental trials are conducted in The Netherlands, Germany, and Austria. Crambe seed oil has a slightly higher proportion of erucic acid (i.e., 55-60%) as compared to rapeseed oil. Since the fibre-rich hull accounts for ca. 30% of the pod weight and usually sticks to the seed, the harvested product contains only some 30% oil. Furthermore, limited genetic diversity in available germplasm of *C. abyssinica* has hindered major progress in the improvement of yield, resistance to insect pests and diseases, as well as meal quality (Vollmann and Ruckenbauer, 1993; Mastebroek et al., 1994). The latter is mainly affected by the presence of glucosinolates, which diminishes the value of the meal as a stock feed (Liu et al., 1993).

White or yellow mustard (*Sinapis alba*), usually used as a spice or condiment crop, has been proposed as another summer-annual cruciferous crop suitable for the production of erucic acid. Several breeding programmes have been started in Sweden and Germany, and more recently in Canada and Israel. *S. alba* is easily grown, resistant to insects, diseases and seed shattering, and requires only a short growing season. But both oil content (30-35%) and seed yield are comparatively low. Nevertheless, *S. alba* has superior drought tolerance in comparison to *B. napus* and is therefore well adapted for production under dryland conditions (Olsson, 1984; Yaniv et al., 1994; Lühs and Friedt, 1994b). As compared to crambe variation for both high oil and low glucosinolate content is not limited, so that development of high erucic, low glucosinolate *S. alba* is feasible (Raney et al., 1995).
Both crambe and yellow mustard have the advantage not to interfere with domestic production of double-low rapeseed in Europe and Canada. Since both crops yield less than only half of the yield achievable from winter oilseed rape (ca. 1200 kg erucic acid per hectare), they cannot be considered as a reliable commercial source of high-erucic oils (cf. Table 1).

Due to its higher seed yield and oil content the raw material for C22 oleochemistry is derived mainly from traditional rapeseed cultivars containing about 45-50% erucic acid in their seed oil. Breeding HEAR has revived and a couple of newly bred cultivars have been registered since 1991. Low glucosinolate content as a surplus trait is realized both in spring and winter type varieties, such as 'Hero' and 'Mercury' (CAN), 'Industry' and 'Erica' (DK) and 'Erox' in Germany. Until now, HEAR breeding has been concerned more with the improvement of seed and oil yield than with the increase of erucic acid content. This causes some quality problems under practical growing conditions. Due to unfavourable climatic conditions such as drought or high temperature during ripening, C22:1 contents less than 45% most frequently are obtained. It is obvious that such a seed oil, bearing only less than a half of the desired fatty acid, is not a very useful raw material for most industrial applications. In order to obtain a rapeseed oil which is more preferable as a raw material for industry, breeders, biochemists and genetic engineers are attempting to maximize the proportion of erucic acid by genetic modification. Actually, numerous projects with this aim are underway, but biochemical constraints are limiting fast success.

Strategies in breeding of HEAR

The use of doubled-haploid (DH) lines

Breeding for modified fatty acid composition in rapeseed (*B. napus*) using classical breeding methods, e.g. pedigree selection, would take at least 10 to 12 years from the original cross to the registration of a new cultivar. Faster breeding progress can be achieved by application of haploid techniques such as microspore culture for the production of doubled-haploid (DH) lines (cf. Thierfelder et al., 1993; Kontowski and Friedt, 1994; Lühs and Friedt, 1994d).

In the course of a breeding programme crosses were carried out between inbred lines which were derived from winter rapeseed cultivars comparatively rich in erucic acid. Both, the parental lines and the F_1 progeny were used as donor plants for microspore culture. The haploid plants were vernalised and treated with colchicine for chromosome doubling in order to get adequate seed material from a large number of DH lines. A choice of 31 selected DH lines was tested for agronomic performance in replicated yield trials at two locations in 1993 and 1994 (Table 1; cf. Lühs and Friedt, 1994d).

TABLE 1. Field performance and quality of selected doubled-haploid (DH) lines and comparative cultivars (00, ++) in a replicated 6x6 lattice field trial #

Genotype	Oil (%)	C22:1 (%)	Yield (dt/ha)	C22:1 yield (kg/ha)
'Lirajet' (00)	39.9	5.2	47.11 a	98
'Falcon' (00)	39.9	5.5	50.51 a	111
'Askari' (++)	43.6 a	54.5 a	45.09 a	1091 a
'Synra' sf 08 (++)	39.0	55.6 a	32.78	723
'Marcus' sf 11 (++)	42.9 a	56.4 *	41.24 a	1010 a
K19-162 (DH)	42.9 a	56.7 *	41.55 a	1007 a
K26-19 (DH)	43.4 a	58.0 *	48.08 a	1215 a
K26-52 (DH)	43.1 a	58.6 *	40.74 a	1032 a
K26-96 (DH)	43.2 a	58.8 *	44.89 a	1144 a
K26-299 (DH)	43.3 a	58.6 *	41.58 a	1058 a
K26-313 (DH)	43.5 a	57.8 *	44.01 a	1109 a
K84-215 (DH)	43.7 a	56.1 a	41.52 a	1025 a
LSD 5%	1.0	1.9	5.84	168
Minimum ##	36.3	1.9	15.44	41
Maximum ##	45.5	60.6	58.33	1474
Mean (overall, n=432)	41.8	53.8	38.47	862

#: mean values of 4 environments (2 years, 2 places) and 3 replications; every parameter on a basis of 91% dry matter content; ##: single value (n=432); 00: double-low quality; ++: HEAR with normal content of glucosinolates; a: no significant differences between the tested genotype and the check cv. 'Askari' (at $p<0.05$ according to LSD test); *: mean value significantly higher than 'Askari' (at $p<0.05$, LSD test).

With regard to oil content, yield and erucic acid yield some of them, especially DHs derived from the cross K26, were competitive with both commercial HEAR- and double-low cultivars. But most of the DH lines possess the advantage of being more suitable for industrial usage due to their elevated erucic acid content (up to 60%).

Selection in vitro in segregating populations of microspore-derived embryoids

Microspore-derived embryoids (MDEs) of *B. napus* have been shown to accumulate storage lipids in a similar fashion than developing rapeseeds, i.e., in the very late cotyledonary stage, the fatty acid composition of MDEs, zygotic embryos and mature seeds are nearly identical. For this reason MDEs have become a suitable research tool in a variety of biochemical and molecular investigations (cf. Taylor et al., 1990; Chen and Beversdorf, 1991; Wiberg et al., 1991; Weber et al., 1992; Taylor and Weber, 1994). For instance, MDEs have been used to evaluate the appearance and regulation of storage compounds (lipids, proteins), and specifically to examine the biosynthesis of triacylglycerols (TAGs) containing high proportions of erucic acid. Since this fatty acid is confined almost exclusively to the neutral lipid fraction in developing rapeseed, it is an ideal marker for the onset of storage lipid synthesis (Taylor et al., 1991, 1992a; Holbrook et al., 1992). Due to their high enzyme activity homogenates of MDEs are capable of TAG bioassembly and it has been demonstrated that they are able to synthesize trierucin if 1,2-dierucoylglycerol and erucoyl-CoA are provided (Taylor et al., 1992b).

With regard to breeding rapeseed oil with a particular fatty acid composition MDEs have been studied in order to select desirable MDEs in segregating populations according to the half-seed method described by Thies (1971). For this purpose the culture conditions of MDE systems have to be optimized in order to synchronize characters of embryoid development (e.g., size, age, ploidy level) and fatty acid biosynthesis on the scale of single MDEs (Wiberg et al., 1991; Albrecht et al., 1994, 1995; Möllers et al., 1994). Although the accumulation of erucic acid is consistently lower in a very late cotyledonary stage of MDEs than in either comparable zygotic embryos or mature seeds (Pomeroy et al., 1991; Taylor and Weber, 1994), Albrecht et al. (1994) have stated that in breeding programmes this effect might not impair the merit of *in vitro* selection in segregating populations. With regard to high erucic acid content the majority (about 70%) of the MDEs can be discarded at an early stage *in vitro* due to the high correlation ($r^2=0.82$) between the eicosenoic + erucic acid content determined in dissected cotyledons of MDEs and that of seeds harvested from plants derived from regenerating embryoids (Albrecht et al., 1995).

Development of novel fatty acid variation by the aid of biotechnology

Brassica napus is a natural amphidiploid species which originated from spontaneous hybridization of *B. rapa* and *B. oleracea*. Although there is new evidence that natural *B. napus* (e.g., oilseed rape, swede and some fodder crops) has arisen by a number of independent hybridization events (Song and Osborn, 1992; Song et al., 1993), the genetic base of this species is rather limited. Contrary, *B. oleracea* and *B. rapa* are both highly polymorphic, since they include important vegetable, oilseed and fodder crops spread all over the world. Therefore, the parental diploid species offer a

much larger variability that can be exploited for *B. napus* improvement via resynthesis, i.e., experimental hybridization of the original progenitors. During the past 40 years, there have been numerous efforts in exploring new germplasm resources and developing breeding stocks by using novel *B. oleracea* x *B. rapa* combinations for resynthesis (cf. Olsson, 1986; Kräling, 1987; Chen and Heneen, 1989; Thierfelder et al., 1993; Prakash and Chopra, 1993; Friedt and Lühs, 1994; Engqvist and Becker, 1994). This favourable route in breeding rapeseed has been further supported by recent investigations using allozyme and molecular markers, in which it has been shown that synthetic *B. napus* lines are often genetically intermediate between their parental diploid species but are very different from the natural *B. napus* forms (Song et al., 1993; Lydiate et al., 1993; Becker et al., 1995).

Progress in breeding for modified fatty acid composition in rapeseed (*B. napus*) using classical breeding methods depends on sufficient variation towards the property of interest. Regarding high erucic acid content several screening programmes have indicated that *B. napus* is limited in having seed oils with erucic acid proportions above 55%. Most of the traditional and currently available oilseed rape cultivars possess an erucic acid content ranging from 45 to 50% (Mahler and Auld, 1988; Lühs and Friedt, 1994c, 1995a). With the aim to select both, high levels of erucic acid and low glucosinolate contents, Ishida (1995) has found considerable variation in about 850 accessions of Japanese rape.

With regard to the seedoil composition of the ancestral diploid species a higher degree of variation has been described for high erucic acid content ranging from 30.1 to 61.4% in *B. rapa* and from 28.2 to 63.4% in the *B. oleracea* cytodeme (including also wild relatives), respectively. Especially the cauliflower seed samples showed the highest proportions of erucic acid ranging from 46.6 to 63.4% with a mean ($\pm$S.E.) of 57.9$\pm$0.3% (Lühs and Friedt, 1995a). Following a biotechnological approach resynthesized rapeseed (*B. napus*) has been generated via wide hybridization using *B. rapa* and *B. oleracea* forms with high erucic acid content (about 55-60%) as crossing parents. Genetic studies involving the newly synthesized rapeseed material have revealed that the genes capable for erucic acid synthesis display an allelic contribution to the total erucic acid content of ca. 16-17% C22:1. However, the opportunity to improve erucic acid synthesis by accumulation and combination of highly effective alleles through resynthesis is obviously limited due to restrictions in the formation of erucic-acid enriched triacylglycerols, especially the synthesis of trierucin (Lühs and Friedt, 1994d, 1995b).

Further, within the crucifer family asexual hybridization through protoplast fusion has become a promising technique for the introgression of desirable traits (e.g., resistance to drought, shattering, pests or diseases) from wild relatives into domesticated brassicas such as *B. napus* or *B. juncea*. Somatic hybrids have been developed between species belonging to the same genus (i.e., *Brassica*) as well as between species from different genera (for review see Vamling and Glimelius, 1990; Sjödin, 1992; Chopra et al., 1993). Much less has been reported concerning more distantly related species combinations. Recently, fertile intertribal hybrids between *B. napus* and *Arabidopsis thaliana* (Forsberg et al., 1994) as well as between *B. napus* and *Thlaspi perfoliatum* (Fahleson et al., 1994) have been obtained by using protoplast

fusion. Although fatty acid inheritance is not a typical cytoplasmic trait like male sterility, resynthesis of *B. napus* has also been accomplished through somatic hybridization in order to induce novel fatty acid variation (Heather and Earle, 1991; Hansen and Earle, 1994). Several research groups have attempted to modify the long-chain fatty acid profile of *B. napus* by means of asexual hybridization between high-erucic acid rapeseed and a range of distantly related crucifers including *Thlaspi perfoliatum* and the ornamental plant *Lunaria annua* (honesty). However, the high level of about 20% nervonic acid found in the seed oil of both of these wild species could not be detected in the progeny of the intertribal hybrids, yet (Fahleson et al., 1994; Craig and Millam, 1995; Möllers, pers. comm.).

Molecular markers and their use in breeding of oilseed rape

Once a useful trait has been identified in a basic breeding stock (e.g., mutant line or germplasm from a wild relative), it may take many years to transfer this character into an elite agronomic background. During the past few years marker-assisted selection is beginning to have a significant impact on the efficiency of plant breeding routines such as backcrossing programmes. Out of the wide range of currently available biochemical and molecular markers, RFLPs, RAPDs and microsatellites (short tandem DNA repeats) are likely to have the greatest effect in crop improvement programmes (Paterson et al., 1991; Waugh and Powell, 1992; Wang et al., 1994; Quiros et al., 1994; Szewc-McFadden et al., 1994). Recently, the two genes controlling erucic acid synthesis in rapeseed were mapped on distinct linkage groups using an RFLP linkage map basing on double-haploid lines derived from the *B. napus* cross 'Mansholts Hamburger Raps' x 'Samourai' (Uzunova et al., 1994, 1995). Suitable markers linked to the genes could be identified that are good candidates for marker-assisted selection of erucic acid content in rapeseed breeding programmes. In the same segregating population three QTLs (quantitative trait loci) for seed oil content could be mapped, of which two QTLs correspond closely to the map positions of the erucic acid genes, indicating a direct effect of the erucic acid genes on seed oil content as mentioned already by Klassen (1976).

Genetic engineering of rapeseed oil with regard to trierucin

Although erucic acid is basically the main constituent of the rapeseed triacylglycerols, it has been shown that this very long chain fatty acid (VLCFA) is confined almost exclusively to the *sn*-1 and *sn*-3 positions of the glycerol backbone. Erucic acid is virtually excluded from the central position. Consequently, no trierucin is found in rapeseed oil, and that means also that the theoretical limit for erucic acid content in rapeseed oil is 66%. Actually, a maximum of approx. 60-63% erucic acid is achievable in cruciferous seed oils, especially in cauliflowers (cf. Lühs and Friedt, 1994c, 1995a). This biochemical limitation could be attributed to both, a reduced capacity of erucic acid synthesis as such and to the unique properties of the lysophosphatidic acid acyltransferase (LPA-AT), the responsible enzyme for the installation of acyl-CoA moieties in the *sn*-2 position. Hence, in the context of increasing the erucic acid content in rapeseed to a maximum, these two biosynthetic pathways emerge as the privileged target sites for genetic modification (Bernerth and Frentzen, 1990; Créach et al., 1993; Taylor et al., 1993).

Recently, again cauliflower genotypes have been identified being able to esterify erucic acid into the central position of the triacylglycerols (Taylor et al., 1994). However, trierucin has not been found in the seed oil of these promising *B. oleracea* mutants. The reasons are unknown and further investigations are indispensable, therefore.

Meanwhile, molecular biologists are attempting to generate trierucin biosynthesis by exchanging the rapeseed LPA-AT for the corresponding enzyme from appropriate donor species, such as meadowfoam (*Limnanthes douglasii*, *L. alba*) which has shown to possess a preference for erucoyl-CoA as substrate (Wolter et al., 1991, 1995; Taylor et al., 1993; Hanke et al., 1994a, 1994b).

TARGET 2: RAPESEED OIL AS AN ANNUAL SOURCE OF LAURICS

Vegetable oils with a high content of lauric acid (C12:0) are widely used raw materials in the manufacture of soaps, shampoos and cosmetic products, detergents and many different surface-active agents, including industrial lubricants, coatings, plastics and other specialty products, e.g., oilfield and mining chemicals. Especially, due to the trend in exploiting renewable resources, the laundry detergent industry dominates the increasing consumption of medium-chain length surfactants. The demand for lauric oils is met almost entirely by palm kernel and coconut oil (cf. Lühs and Friedt, 1994a, 1994b).

In the past few years, several projects have been initiated in order to increase the availability of laurate and other medium-chain fatty acids (C10 to C14) by genetic engineering of rapeseed. Scientists of Calgene Inc., Davis, California, have successfully demonstrated that a chain termination mechanism, a C12:0 specific acyl-[ACP] thioesterase transferred from California bay (*Umbellularia californica*) into rapeseed, is responsible for the accumulation of about 45-50% lauric acid in the storage triacylglycerols of transgenic rapeseed. Since high-laurate canola is subjected to similar constraints as mentioned above for the biosynthesis of trierucin, the Calgene genetic engineers expect to maximize the lauric acid content through introduction of a coconut LPA-AT gene into the laurate canola lines (Voelker et al., 1992; Knauf, 1994; Davies et al., 1994).

Further attention has been focused on species in the genus *Cuphea* of the family *Lythraceae*. The seeds of these herbaceous species most commonly have 30-33% oil, which is a rich source of fatty acids ranging from caprylic (C8:0) to myristic acid (C14:0). In particular, one important advantage of *Cuphea* seed oils as compared to traditional sources of lauric oils is that high proportions (up to 95%) of specific single fatty acids, such as C8:0 or C10:0, are feasible (Graham and Kleiman, 1992). Recently, several acyl-[ACP] thioesterase cDNAs and genes from *C. lanceolata*, an annual plant which predominantly contains capric acid (C10:0, up to 83%) in its seed oil, have been expressed in *Brassica napus*. Due to more preliminary results it has been shown that the C8 to C18 fatty acid pattern (calculated on a mol% basis) of rapeseed oil is drastically changeable, since in the mature T2 seeds of one transgenic plant 1% C8:0 and 3% C10:0 as well as 7% C14:0 and 15% C16:0 in another one were detected (Töpfer and Martini, 1994; Martini et al., 1995).

CONCLUSIONS

Breeding oilseed rape with very high proportions of erucate or laurate - possibly more than 80% for usage as a high-grade industrial oil - is limited by the fact that rapeseed oil is lacking trierucin and lauric acid as such. It is obvious that this goal - the development of novel rapeseed cultivars capable of producing seed oils tailor-made for industrial purposes - is attainable only by the aid of genetic engineering rather than by conventional breeding procedures (e.g., mutation breeding, interspecific or intergeneric hybridization, pedigree selection, or hybrid breeding). Trierucin is predicted to provide the main breakthrough in breeding and production of high-erucic acid oils, because it would make the processing of comparatively pure erucic acid much easier and more attractive commercially. Rapeseed oil as an annually growing domestic source of laurate would relax the dependence upon imports and it would be easier to adjust supply to demand, which could stabilize the price of lauric oils for end users. However, the development of trilaurin will be necessary to make Calgene's laurate canola more competitive, because it currently contains a level of lauric acid which is only comparable to coconut and palm kernel (about 50% C12:0).

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