Genetic Control of Fatty Acid Biosynthesis in Rapeseed (*Brassica napus* L.)[1]

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Abstract

Isolation of rapeseed plants containing no erucic acid in their seed oil gave simultaneous selection for low eicosenoic acid. Genetic analysis of populations segregating for erucic acid content indicated that the synthesis of these fatty acids was controlled, in the developing embryo, by two genes which displayed no dominance and acted in an additive manner. As the genetic capacity for erucic acid synthesis decreased, there was an increase in percentage of oleic acid with no corresponding decrease in total oil content. The percentage of eicosenoic acid remained relatively constant with decreasing erucic acid except in the zero erucic acid genotype where only 1% eicosenoic was detected instead of approx 12%. These observations were interpreted to mean that eicosenoic and erucic acids were formed by a genetically controlled carbon chain lengthening system operating by the addition of acetate molecules to the carboxyl end of oleic acid. This hypothesis was supported by data obtained from the injection of radioactive sodium acetate into immature rapeseed pods. In the monoene fraction of the oil, eicosenoate had three times the specific activity of oleate and erucate had twice the activity of eicosenoate. On oxidation, the monocarboxylic fragments of these three acids had low and similar specific activities while in the dicarboxylic acids, where chain elongation has presumably taken place, activity increased in the ratio of 1:5:19.

Introduction

Rapeseed has become a major agricultural crop in Western Canada with an annual production of over 250,000 tons of seed. The major portion of this seed has been exported to European and Asian markets but domestic demand for the oil as an edible product has been increasing. Research has been underway for a number of years to improve oil quality and to modify the fatty acid composition through plant breeding.

The fatty acid composition of rapeseed oil differs from other vegetable oils in the presence of major amt (9–15%) eicosenoic and (20–45%) erucic acids and minor amt (1–2%) arachidic, behenic and lignoceric acids (1,3,13). Previous investigations on the effects of variety and environment have shown highly significant linear relations between oleic and erucic acids (2).

Selection within the species *Brassica napus* L. for plants with low erucic acid resulted in a strain which contained no erucic acid in its seed oil (9). Reciprocal crosses between these plants and normal high erucic acid types demonstrated that fatty acid composition was controlled by the genetic constitution of the embryo rather than that of the maternal parent (5). Genetic analysis of the F_2, F_3 and backcross seed supported the hypothesis that the erucic acid content of the seed oil was controlled by two genes. No genetic dominance was found; the genes acted instead in an additive manner, each contributing 9–10% erucic acid to the oil under the conditions used in these studies (6).

We have observed that the percentage of oil in different rapeseed varieties was relatively constant, despite wide variation in fatty acid composition. This was suported by the further observation that plants containing no erucic acid likewise produced a percentage of oil similar to standard varieties. One explanation is that the C_{16} and C_{18} fatty acids are synthesized in a manner similar to other plant systems, whereas the monounsaturated C_{20} and C_{22} acids result from a chain elongation system similar to that shown by Wakil (12) and Mead (7) in animal tissues. The proposed system would involve oleic acid as the precursor and the addition of one acetate to the carboxylic end of the fatty acid molecule to form eicosenoic acid and two acetates to form erucic acid.

This system would satisfy two requirements: a) the unsaturated positions in the proposed scheme would be those found in the C_{20} and C_{22} monoene acids in rapeseed oil, and b) the total fatty acids produced would not be affected since the change in composition would involve an acid already formed.

The present study was undertaken to test the proposed biosynthetic scheme by feeding C^{14} labelled acetate to maturing rapeseed. If a chain elongation system is present which incorporates acetate directly, then the C_{20} and C_{22} fatty acids should reflect this change by high specific activities, with the activity located in the carboxylic end of the fatty acid chains.

The study also deals, in part, with changes in other fatty acids of rapesed oil resulting from the reduction and elimination of erucic acid, and their implications to the biological pathway of fatty acid synthesis.

Methods and Materials

To show the relationship between fatty acid composition of the seed oil and total oil content, seed of *B. napus* with genotypes capable of producing five levels of erucic acid were identified by using the half seed technique of Downey and Harvey (5). These seeds were germinated and the plants grown to maturity in a growth chamber maintained at 24C day, 15.6C night temp, and a day length of 18 hr. Self-pollinated seed harvested from each plant was oven-dried, weighed and crushed with a glass rod in a 50-ml Erlenmeyer flask containing 10 ml solution of methanol, acetyl chloride and benzene in the ratio of 20:1:4. The mixture was refluxed under an air condenser for 1 hr to extract and esterify the seed oil. Filtration of the sample through glass wool into a 50-ml flask with the bottom drawn to a point, followed by evaporation on a rotary evaporator, under reduced pressure, removed the seed particles and concd the sample. A known wt of internal standard (dibutyl sebacate dissolved in carbon tetrachloride) was added and 0.2–0.4 μl of the sample injected into an F and M model 500 gas chromatograph operated at 208C with a helium flow rate of 75 ml/min and using an 8 ft × 3/16 in. ID copper column containing

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TABLE I

Oil Content and Fatty Acid Composition of Self-pollinated Seeds from Rape Plants Genetically Capable of Producing Five Levels of Erucic Acid

Plant genotype [a]	Seed oil content, %	Fatty acid composition in percentage of total acids								
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:1
++++	39.0	3.8	0.4	0.8	18.4	15.8	9.8	0.8	11.0	39.2
−+++	45.5	3.8	0.5	1.3	24.3	15.7	9.1	1.0	13.9	30.4
−−++	38.9	4.1	0.4	1.4	34.2	16.3	7.9	0.7	14.1	20.9
−−−+	39.4	4.8	0.7	0.8	41.8	18.7	7.3	1.0	12.1	12.8
−−−−	43.4	4.8	0.9	1.2	63.1	20.0	8.2	0.8	1.0	0.0

[a] (+) indicates a gene contributing 9–10% erucic acid to the seed oil.

42–60 mesh chromosorb W with butanediol succinate in the ratio of 8:1 (w/w). The oil content of each sample was calculated by determining peak areas by triangulation and relating the total fatty acid peak area to the peak area of the internal standard.

Rapeseed plants of *B. napus* bearing pods, which were judged to be at the stage of half maturity based on oil content, were selected from a field stand. Sixty-seven pods were excised from the branches of the plants and placed base down in a beaker containing sufficient distilled water to cover the pedicle of the pods. At the base of each pod, 10 µl aqueous solution of radioactive sodium acetate (0.2 µc methyl labelled) was injected with a Hamilton micro-syringe. A branch from a rapeseed plant bearing 15 pods was excised below the lowest pod, the pods were similarly injected and the cut end of the branch placed in a beaker partially filled with distilled water. All cutting operations were carried out under water to prevent air entrapment. Samples were kept in an artificially lighted chamber maintained at 25C for 24 hr. Oil obtained from the 67 excised pods had an activity of 4,650 c.p.m. at infinite thickness, whereas the oil from the 15 pods attached to the branch showed an activity of 4,800 c.p.m. Since the uptake of radioactive acetate was similar, the oil samples were bulked for further analysis.

The seeds were dried in a vacuum oven, at 85C for 2 hr, and the oil extracted in a Swedish tube (10) using a chloroform-methanol (2:1) solvent system. The solvent was removed under reduced pressure and residue extracted with diethyl ether which also was removed under reduced pressure. The sample was saponified, and unsaponifiable material was separated by extracting the soaps in aqueous ethanol with ethyl ether. The soaps were converted to free fatty acids and esterified with methanol using boron trifluoride-etherate complex as a catalyst (8).

The methyl esters were separated into saturated monoene, diene and triene fractions by silicic acid-silver nitrate column chromatography according to the method of De Vries (4). The column was 25 mm × 450 mm long and contained 60 g packing. Several hundred mg esters were applied to the column—the fractions, solvent systems and volumes being as follows: saturated, 900 ml 10% benzene in Skellysolve "F"; monoenes, 700 ml 40% benzene in Skellysolve "F"; dienes, 800 ml 60% benzene in Skellysolve "F"; and trienes, 600 ml ethyl ether. Fractions were collected at intervals and checked for fatty acid composition by GLC analysis using an *o*-phthalic ethylene glycol polyester on C-22 fire brick (1:4.5 w/w) in an 8 ft × 3/16 in. copper column at 205C and 60 cc/min helium flow rate.

The saturated and monoene fractions were separated into individual esters by GLC using a 3 ft × ¼ in. SE 30 on Chromosorb W (1:6) copper column at 220C and 60 cc/min helium flow rate. Approx

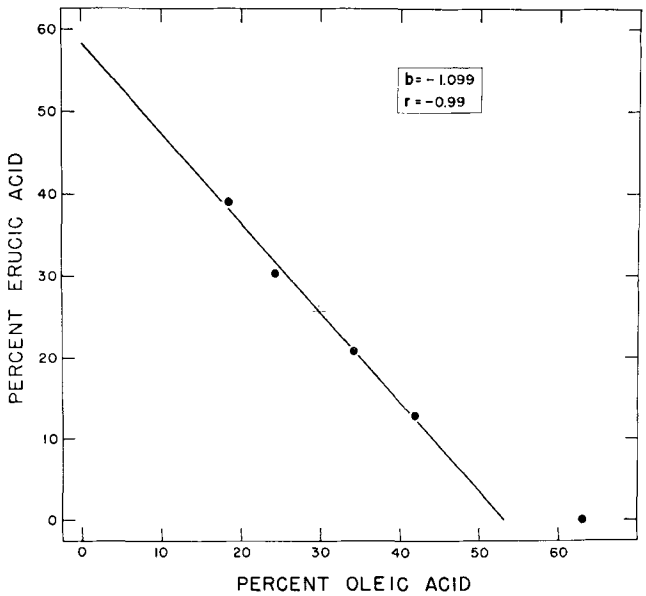

Fig. 1. Relationship between erucic and oleic acids of rapeseed in the genotypes from *Brassica napus* L. producing different levels of erucic acid.

50 µl aliquots of esters were injected and individual fractions were collected in solvent using a cold trap.

The oxidation of the monoene methyl esters, oleate, eicosenoate and erucate were carried out using the permanganate-periodate reagent (11). The short chain monocarboxylic acids were recovered by steam distillation, and the dicarboxylic acids by extraction of the acidified dried residue using chloroform as a solvent.

Radioactivity measurements were carried out on all fractions in an ionization chamber (Nuclear Chicago, Dynacon Electrometer Model 6,000) after wet combustion of a known wt of sample.

Results

Selection for low erucic acid in the seed oil of *B. napus* gave no significant change in total oil content despite the displacement of approx 40% erucic acid and 10% eicosenoic acid (Table I). The inverse relationship between erucic and oleic acid apparent in these data confirm the findings of Craig (2) on differences in fatty acid composition of the oil of rapeseed varieties. However, only the first four genotypes show a linear relationship ($r = -0.98$) between oleic and erucic acid (Table I, Fig. 1). The regression equation calculated from these data was erucic acid % $= -1.099$ (oleic acid %) $+ 58.44$ SE $\pm$ 1.0%. The genotype which produced no erucic acid showed a marked deviation from the linear relationship. Fatty acids, other than the monoethenoid acids, oleic, eicosenoic and erucic, remained relatively constant in all genotypes, with the possible exception of linoleic acid which showed some increase in the low and zero erucic acid genotypes.

The separation of 1.43 g methyl esters of the rapeseed fatty acids, according to the degree of saturation, was done on two silicic acid-silver nitrate columns which gave identical results as judged by GLC analysis of the eluates. The saturated and monoene fractions were pure; however, the first diene fraction showed some carryover of monoene acids and was therefore separated from the second diene fraction which was relatively pure linoleic acid (Table II). The triene fraction was mainly linolenic acid. On analysis of all fractions the highest specific activities

TABLE II

Specific Activity and Fatty Acid Composition of Fractions of Rapeseed Methyl Esters Resulting from Silicic Acid-Silver Nitrate Column Chromatography

Fraction	Wt g	Spec. act. mμc/mg	Fatty acid	% Comp. by GLC
Saturated	0.18	0.63	14:0	0.9
			16:0	48.7
			18:0	26.0
			20:0	15.7
			22:0	8.7
Monoene	0.91	0.84	16:1	0.4
			18:1	35.2
			20:1	22.6
			22:1	41.8
Diene I	0.14	0.25	16:1	0.8
			18:1	11.1
			18:2	84.3
			20:2	3.8
Diene II	0.11	0.30	18:1	0.7
			18:2	98.4
			20:2	0.9
Triene	0.12	0.22	18:2	1.5
			18:3	97.5
			20:3	1.0

were recorded in the monoene and saturated fractions (Table II).

The monoene fraction was separated into the four individual methyl esters by GLC and their specific activities determined (Table III). Methyl oleate, eicosenoate and erucate showed increased activities corresponding to chain length. Good agreement was obtained between the percentage of composition on the basis of wt of methyl ester recovered (Table III) and the original GLC analysis of the mixture of esters (Table II).

The saturated fraction was also separated by GLC into the four individual esters and the specific activities in mμc/mmole were as follows: palmitate 56.8, stearate 131.3, arachidate 320.0 and behenate 677.3. Thus the trend was to higher activities with longer carbon chains.

The monoethenoid esters were then oxidized to yield the monocarboxylic and dicarboxylic acids and the specific activities of these scission products determined (Table IV). The specific activities of the monocarboxylic fragments were low and of the same order of magnitude. However, large differences were recorded among the dicarboxylic fragments.

Discussion

It appears that the genes controlling production of eicosenoic and erucic acids have no direct effect on total fatty acid production since contrasting homozygous genotypes produced similar amt of oil despite marked changes in fatty acid composition (Table I).

The palmitic, palmitoleic, stearic, linolenic and arachidic acids showed no regular variation throughout the genetic series. However, linoleic tended to increase with increasing amt of oleic acid.

The linear relation between erucic and oleic acids (Fig. 1) is similar to the relationship found in a varietal study (2). The prediction equation from the present data: Erucic acid % = −1.099 (oleic acid %) + 58.44 SE ± 1.0 indicates that in the material under study the max level of erucic acid that could be obtained by selection would be 58.4%. Higher erucic acid values might be obtained if part or all the eicosenoic acid was converted to erucic acid. If the prediction equation is calculated in the form oleic acid % = −0.9097 (erucic acid %) + 53.17 the level of the oleic acid which would be expected when the erucic acid level is zero would be 53.2%. The value 63.1% oleic acid found in the zero erucic genotype represents the oleic acid expected plus the 10% which would normally be converted to eicosenoic acid.

TABLE III

Amounts and Specific Activities of the Fatty Acid Esters Obtained by GLC Separation of the Monoene Fraction

Fatty acid ester	Spec. act. mμc/mmole	Recovered wt mg	Wt %	GLC %
Palmitoleate	163.8	1.4	0.3	0.5
Oleate	71.2	123.4	35.0	35.8
Eicosenoate	233.6	77.0	21.8	21.5
Erucate	468.8	151.1	42.9	42.2

In the previous varietal study (2) the calculated prediction equation was oleic acid % = −.8590 (erucic acid %) + 53.61 SE ± 1.4%. This equation is not significantly different from that derived from the four erucic acid genotypes. In addition, the overall regression coefficient and individual varietal regressions were not significantly different, which suggests that it should be possible to select a similar range of erucic acid from each of the six varieties used in the previous study.

The maintenance of eicosenoic acid at a level of 11–14% of the total acids in all but the zero erucic acid genotypes supports the hypothesis that the synthesis of erucic acid is a stepwise process and that eicosenoic acid is an intermediate between oleic and erucic acids in the chain elongation system.

The high specific activity of the monoene fraction of the radioactive methyl esters and the marked increase in activity found in eicosenoic and erucic acids (Tables II, III and IV) strongly supports the assumption that the radioactive acetate had been incorporated into these molecules. This assumption was substantiated by oxidation of the monoene acids. On oxidation each acid yields the same monocarboxylic acid and a different dicarboxylic acid. If oleic acid is the precursor, then the monocarboxylic ends should have ca. the same radioactivity, and in the dicarboxylic ends additional radioactivity would represent chain elongation. This is essentially what was recorded (Table IV). The monocarboxylic acids remained relatively constant, whereas the dicarboxylic end of eicosenoic acid showed five times the radioactivity of oleic, and that of erucic ca. four times the radioactivity of eicosenoic.

The radioactive study substantiated the proposed mechanism for the biosynthesis of eicosenoic and erucic acids through chain elongation of oleic acid by the addition of acetate molecules. The monoene fraction is the most radioactive, followed by the saturated fraction (Table II). The diene and triene fractions, composed of approx 98% linoleic and linolenic acids, have the same activity as the oleate (Table II). The three monoene esters, oleate, eicosenoate and erucate (Table III) show specific activities in the order of 1:3:6. Further on oxidation the monocarboxylic portions of these three monoene acids have approx the same activity but the dicarboxylic portions are in the ratio of 1:5:19 in terms of specific activity. Palmitoleic acid shows a higher specific activity than oleic acid, but could not function as a precursor in a chain elongation system because of the position of the unsaturated bond in the hexadecenoic acid.

TABLE IV

Specific Activities and Chain Length of Mono- and Dicarboxylic Acids Produced by Oxidation of Monoene Esters

Fatty acid	Specific activity mμc/mmole	
	Monocarboxylic	Dicarboxylic
9—Oleic	C_9 26.9	C_9 20.7
11—Eicosenoic	C_9 33.2	C_{11} 101.5
13—Erucic	C_9 26.9	C_{13} 388.0

The increasing activity of the saturated acids with increasing chain length suggests that a similar chain lengthening mechanism may operate in the synthesis of saturated fatty acids. The radioactivity of each saturated acid was approx double that of the previous acid. This might be interpreted as a series, starting with palmitic acid as the precursor. However, if this is true, the biosynthetic system must be under a control distinct from that for the monoene acids; oleic, eicosenoic and erucic, since no changes in the relative amt of the saturated acids were recorded in fatty acid composition of the high and zero erucic acid oils (Table I). If the chain lengthening mechanism for the saturated acids was under the same control as for the monoethenoid acids, then no stearic, arachidic or behenic acids would be expected in seed from the zero erucic acid plants. Thus a chain lengthening system for the saturated acids is not precluded but, if it exists, evidently it is not identical to that proposed for the monoethenoid acids.

It is interesting to note that, under the conditions of the present study, the specific activities for oleic, linoleic and linolenic acids (Table II) are approx equal and are each lower than stearic acid.

The results of this study show the presence of at least two separate biosynthetic pathways in rapeseed fatty acid biosynthesis. The chain lengthening system under direct genetic control operates to add an acetate molecule to the carboxylic portion of oleic acid to form eicosenoic acid, and the addition of a second acetate molecule to form erucic acid. This genetic control is indicated by the fatty acid composition of the oil from the five genotypes studied, where the substitution of a (−) gene for a (+) gene reduced the amt of erucic acid by 9–10% under the experimental conditions used (Table I). A similar chain-lengthening system has been shown for the formation of archidonic acid in animal tissue where linoleic acid is the precursor, although genetic control has not been demonstrated in this animal system (7). It is of interest to speculate on the type of genetic control operating in the plant system. It is possible that the genes control the enzymes which regulate the production of substrates or precursors necessary for the synthesis of eicosenoic and erucic acids or it may be that the genes control the quantity of enzyme produced for the chain elongation system. These possibilities are under further investigation and will be the subject of future publications.

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Two New Stable Polybrominated Salicylanilides for Antibacterial Use in Soap and Detergent Products

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Abstract

The versatility of two brominated salicylanilides as antiseptic agents and germicides in soap and detergent products for use on skin, hair, hard surfaces and in fabrics is discussed.

Both preparations show stability at elevated temp and pressure and compatibility with many surfactant vehicles. They are free of primary irritation and sensitization, including sunlight-induced irritation.

A mixture of di- and tribromosalicylanilide is currently being used in toilet bars, shampoos, laundry products, disinfectant sprays and multi-purpose disinfectant products. The substantivity to fabrics is further characterized by stability to heat and hydrolysis by alkalinity.

Essentially pure 3,4′,5 tribromosalicylanilide is resistant to discoloration, making it particularly suitable for use in white and pastel colored toilet bars. It has excellent substantivity to skin and provides long-lasting action against odor-causing and pathogenic bacteria.

Introduction

ANTIBACTERIAL AGENTS have been finding increased application in household products in recent years. They are being used in toilet soaps, detergent products and hard surface cleansers, as well as personal products such as ointments, creams, lotions, underarm deodorants and shampoos. These germicides have proved effective in sprays and multi-purpose disinfectant cleansers specially formulated to combat the alarming rise of infections of Staphylococcus aureus and other pathogens in hospitals.

Desirable performance specifications of germicides are:

1. Activity at low concn
2. Compatibility with surface active systems and cosmetic vehicles
3. Heat, odor and color stability
4. Sustained residual activity
5. Safety in handling and use; absence of primary irritation, sensitization and photosensitization
6. Substantivity to both animate and inanimate surfaces
7. Economical

Germicides used in toilet soaps include hexachlorophene, bithionol, trichlorocarbanilide and formerly tetrachlorosalicylanilide. Each of these has some shortcoming (1,2) in that not all of the above outlined specifications are satisfied.

The halogenated salicylanilides have been studied quite extensively because of their exceptionally high germicidal activity, as discussed by LeMaire et al. (3). The conformance of certain derivatives of salicylic acid to the above specifications suggests that they are the antimicrobial agents of choice for soap and detergent products. Of the halogenated salicylanilides in-