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(54) Titre : GENES ELONGASE D'ACIDE GRAS (FAE) ET LEUR UTILITE DANS L'AUGMENTATION DE L'ACIDE ERUCIQUE ET AUTRES PROPORTIONS D'ACIDE GRAS A TRES LONGUE CHAINE DANS L'HUILE DE GRAINES

(54) Title: FATTY ACID ELONGASE (FAE) GENES AND THEIR UTILITY IN INCREASING ERUCIC ACID AND OTHER VERY LONG-CHAIN FATTY ACID PROPORTIONS IN SEED OIL

(57) Abrégé/Abstract:
This invention relates to seeds of plant, plants themselves and cells of such plants which comprise a heterologous gene coding for a plant (such as nasturtium (Tropaeolum majus) or Crambe abyssinica) fatty acid elongase (FAE) gene or allelic variant thereof, or combinations of one or both of these FAE genes with an Arabidopsis fatty acid elongase 1 (FAEI) gene, in co-transformation, in reading frame alignment with a promoter capable of increasing expression of said gene(s), when said transformed plant cell is in a seed, said plant cell or seed being capable of producing an increase in proportion of a very long chain monounsaturated or saturated fatty acids when compared with the proportions of said fatty acids in a control plant cell or seed lacking said heterologous FAE gene or genes. The invention also relates to combinations of these fatty acid elongase genes by traditional crossing, sufficient to increase the proportion of very long chain monounsaturated or saturated fatty acids in the seed oil of the progeny compared to the proportion of said fatty acids in either of the parental lines.

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(57) Abstract: This invention relates to seeds of plant, plants themselves and cells of such plants which comprise a heterologous gene coding for a plant (such as nasturtium *(Tropaeolum majus)* or *Crambe abyssinica)* fatty acid elongase (FAE) gene or allelic variant thereof, or combinations of one or both of these *FAE* genes with an *Arabidopsis* fatty acid elongase *1 (FAEI)* gene, in co-transformation, in reading frame alignment with a promoter capable of increasing expression of said gene(s), when said transformed plant cell is in a seed, said plant cell or seed being capable of producing an increase in proportion of a very long chain monounsaturated or saturated fatty acids when compared with the proportions of said fatty acids in a control plant cell or seed lacking said heterologous *FAE* gene or genes. The invention also relates to combinations of these fatty acid elongase genes by traditional crossing, sufficient to increase the proportion of very long chain monounsaturated or saturated fatty acids in the seed oil of the progeny compared to the proportion of said fatty acids in either of the parental lines.

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Fatty Acid Elongase *(FAE)* genes and their utility in increasing erucic acid and other very long-chain fatty acid proportions in seed oil.

Background to the invention

Very long chain fatty acids (VLCFAs) with 20 carbons or more are widely distributed in nature. In plants they are mainly found in epicuticular waxes and in the seed oils of a number of plant species, including members of the *Brassicaceae*, *Limnantheceae*, *Simmondsia* and *Tropaeolaceae*. A strategic goal in oilseed modification is to genetically manipulate high erucic acid (HEA) germplasm of the *Brassicaceae* to increase the content of erucic acid (22:1 Δ13) and other strategic VLCFAs in the seed oil for industrial niche market needs. Erucic acid and its derivatives are feedstocks in manufacturing slip-promoting agents, surfactants, plasticizers, nylon 1313, and surface coatings and more than 1000 patents have been issued. The current market for high erucate oils exceeds $120 million U.S./annum. Worldwide erucic acid demand is predicted to increase from about 40 million pounds (M pds) in 1990 to about 80 M pds by the year 2010. Similarly, demand for the derivative, behenic acid, is predicted to triple to about 102 M pds by 2010. In recent years, production has increased to meet market needs and high erucic acreage in western Canada is currently at a record high. A *Brassica* cultivar containing erucic acid levels approaching 80% would significantly reduce the cost of producing erucic acid and its derivatives and could meet the forecast demand for erucic and behenic acids as renewable, environmentally-friendly industrial feedstocks.

VLCFAs are synthesized outside the plastid by a membrane bound fatty acid elongation complex (elongase) using acyl-CoA substrates. The first reaction of elongation involves condensation of malonyl-CoA with a long chain substrate producing a 3-ketoacyl-CoA. Subsequent reactions are reduction of 3-hydroxyacyl-CoA, dehydration to an enoyl-CoA, followed by a second reduction to form the elongated acyl-CoA. The 3-ketoacyl-CoA synthase (KCS) catalyzing the condensation reaction plays a key role in determining the chain length of fatty acid products found in seed oils and is the rate-limiting enzyme for seed VLCFA production. Hereafter the terms elongase and FAE will signify 3-ketoacyl-CoA synthase condensing enzyme *genes*/proteins. The composition of the fatty acyl-CoA pool available for elongation and the presence and size of the neutral lipid sink are additional important factors influencing the types and levels of VLCFAs made in particular cells.

Our knowledge of the mechanism of elongation and properties of FAE1 and other elongase condensing enzymes is, in part, limited by their membrane-bound nature: as such they are more difficult to isolate and characterize than soluble condensing enzymes. The genes encoding

FAE1 and its homologs have been cloned from *Arabidopsis thaliana* and from *Brassica napus* (two homologous sequences, Bn-FAE1.1 and Bn-FAE 1.2).

Site-directed mutagenesis experiments have been carried out on the *Arabidopsis* FAE1 to decipher the importance of cysteine and histidine as residues in condensing enzyme catalysis. Results have shown that cysteine[223] and four histidine residues are essential for the enzyme activity.

In this work, we selected *Tropaeolum majus*, garden nasturtium, as a source of the elongase involved in VLCFA synthesis based on the fact that this plant is capable of producing significant amounts of erucic acid (70-75 % of total fatty acid) and accumulates trierucin as the predominant triacylglycerol (TAG) in its seed oil. Here, we report the isolation of a nasturtium *FAE* gene and demonstrate the involvement of its encoded protein in the elongation of saturated and especially monounsaturated fatty acids. We also selected *Crambe abyssinica* as a second source of an elongase gene since *Crambe* is grown, particularly in the US, as an alternative crop for high erucic acid 46-50% (wt/wt) oil.

This invention relates to a nasturtium cDNA encoding an "elongase" (condensing enzyme) with a high specificity for eicosenoyl moieties which can be utilized to engineer seed oil crops for production of high erucic acid oils. This invention also relates to a *Crambe* cDNA encoding an elongase with a strong capability to synthesize erucic acid.

There is interest in modifying the seed oil fatty acid composition and content of oilseeds by molecular genetic means to provide a dependable source of Super High Erucic Acid Rapeseed (SHEAR) oil for use as an industrial feedstock.

Nonetheless, to date, increases in the content of some strategic fatty acids have been achieved by introduction of various fatty acid biosynthesis genes in oilseeds. Some examples include:

Expression of a medium chain fatty acid thioesterase from California Bay, in Brassicaceae to increase the lauric acid content. (Calgene)

Expression of an anti-sense construct to the Δ9 desaturase in Brassicaceae to increase the stearic acid content. (Calgene)

Increased proportions of oleic acid by co-suppression using constructs encoding plant microsomal desaturases. (DuPont/Cargill)

Expression of a *Jojoba* "elongase" 3-keto-acyl-CoA synthase in low erucic acid (canola) *B. napus* cultivars to increase the level of erucic acid; the effect following expression in high erucic acid cultivars was negligible (Calgene).

However, there has not been an elongase gene identified or characterized as encoding an FAE with the ability to produce 22:1 beyond the level already existing in HEAR*B. napus* cultivars.

We considered that the isolated FAE "elongase" homolog from *Tropaeolum majus* (garden nasturtium) with GenBank Accession No. AY082610 (published on March 6th, 2002), could be used to engineer plants to produce seed oils highly enriched in erucic acid. We found that to date, this is the first "elongase" transgene experiment to result in an 8-fold increase in the proportions erucic acid in *Arabidopsis* plants. When expressed in *B. carinata*, the nasturtium *FAE* gene resulted in an increase in erucic acid proportions of up to 6%. When co-expressed with the *Arabidopsis FAE* gene in *B. carinata* the result was an increase in erucic acid proportions by up to 16-18%.

We also cloned and functionally expressed in yeast, a *Crambe FAE* gene (GenBank Accession No. AY793549), resulting in the accumulation of 20:1 c 11 and 22:1 c13, fatty acids not found in wild-type yeast control lines.

To our knowledge the nearest art relates to an elongase gene (*FAE1*) from *Arabidopsis* which was cloned and published as: James, D.W. Jr., Lim, E., Keller, J., Plooy, I., Ralston, E. and Dooner, H.K. (1995) Directed tagging of the Arabidopsis FATTY ACID ELONGATION1 (FAE1) gene with the maize transposon activator. *The Plant Cell* 7: 309-319 (1995).

The reader is also referred to sequences 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 35, 37, 39, 41 from Jaworski, J.G. and Blacklock, B.J. Patent Application WO0194565 as well as sequences 19, 20, 21, 22, 23 from Kunst and Clemens, Regulation of embryonic transcription in plants. Patent Application WO0111061; 15-FEB-2001; University of British Columbia (CA).

Summary of the Invention

The invention relates to an expression vector for transforming a cell, the expression vector comprising a gene coding for a plant fatty acid elongase in reading frame alignment with a promoter capable of increasing expression of the gene, when the transformed cell is in a seed, sufficient to increase the proportion of very long chain monounsaturated fatty acid when compared with a control cell. The expression vector may, for example, comprise a gene encoding a nasturtium (*Tropaeolum majus*) fatty acid elongase gene, a *Crambe* fatty acid elongase gene or an *Arabidopsis* fatty acid elongase 1 (*FAE1*) gene. The expression vector may further, for example, comprise a gene encoding a nasturtium (*Tropaeolum majus*) fatty acid elongase gene or a *Crambe* fatty acid elongase gene, or combinations of one or both of these *FAE* genes with an *Arabidopsis* fatty acid elongase 1 (*FAE1*) gene in co-transformation experiments in reading frame alignment with a promoter capable of increasing expression of said gene(s), when said transformed cell is in a seed, sufficient to increase in proportion of very long chain monounsaturated fatty acid when compared with a control cell. The invention also relates to cell comprising a heterologous gene coding for a heterologous plant fatty acid elongase or allelic variant thereof, said cell being capable of producing an increase in proportion of a very long chain monounsaturated fatty acid when compared a control cell lacking said heterologous gene. The cell may, for example, be a fungal,

yeast or plant cell, especially a plant seed cell. The cell may, for example, comprise a heterologous gene coding for a nasturtium, *Crambe*, or *Arabidopsis* fatty acid elongase gene or allelic variant thereof, said cell being capable of producing an increase, preferably at least a 10% increase, in proportion of a very long chain monounsaturated fatty acid (e.g. erucic acid) when compared with a control cell lacking said heterologous gene. The increase can be larger, e.g. up to about eight-fold. In a plant cell of the invention the heterologous gene may code for a 3-ketoacyl-CoA synthase. The plant cell of the invention may additionally comprise a further heterologous gene coding for an additional heterologous plant fatty acid elongase or allelic variant thereof or a heterologous plant desaturase gene or allelic variant thereof. The plant cell of the invention preferably is capable of producing oil with an increased content of erucic acid or other very long chain fatty acid (C_{20} or greater). The invention also relates to seeds and plants comprising such cells and the use of such vectors to produce such cells, seeds and plants. The plant preferably is a dicotyledon, especially a member of the *Brassicaceae*, *Limnanthaceae*, *Tropaeolaceae* or *Simmondsia*. Plants of the genus *Brassica* and *Linum usitatissimu* L. are especially preferred.

The invention also relates to a method for altering erucic acid content of a plant-derived oil which method comprises cultivating a plant of the invention and then extracting a plant-derived oil therefrom which oil has altered erucic acid content. Use of a heterologous plant fatty acid elongase gene for altering erucic acid content in a plant is also contemplated. Use of a heterologous plant fatty acid elongase gene for altering the very long chain fatty acid content (C_{20} or greater) in a plant is further contemplated.

The fatty acid elongase (often designated FAE or 3-ketoacyl-CoA synthase (KCS)) is a condensing enzyme and is the first component of the elongation complex involved in synthesis of erucic acid (22:1) in seeds of *Tropaeolum majus* (garden nasturtium). Using a degenerate primers approach, a cDNA of a putative embryo *FAE* was obtained showing high homology to known plant elongases. This cDNA contains a 1512-nucleotide open reading frame (ORF) that encodes a protein of 504 amino acids. A genomic clone of the nasturtium *FAE* was isolated and sequence analyses indicated the absence of introns. Northern hybridization showed the expression of this nasturtium *FAE* gene to be restricted to the embryo. Southern hybridization revealed the nasturtium 3-ketoacyl-CoA synthase to be encoded by a small multigene family. To establish the function of the elongase homolog, the cDNA was introduced into two different heterologous chromosomal backgrounds, *Arabidopsis* (*A. thaliana*) and tobacco (*Nicotiana tabacum*), under the control of a seed-specific (napin) promoter and the tandem 35S promoter, respectively. Seed-specific expression resulted in up to an 8-fold increase in erucic acid proportions in *Arabidopsis* seed oil. Constitutive expression in transgenic tobacco tissue resulted in increased proportions of very long chain saturated fatty acids. These results indicate that the nasturtium *FAE* gene encodes a condensing enzyme involved in the biosynthesis of very-long-chain fatty acids, utilizing

monounsaturated and saturated acyl substrates. It shows utility for directing or engineering increased synthesis of erucic acid in other plants. Using a PCR based approach, a cDNA of an embryo-specific *FAE* was cloned from *Crambe abyssinica*.

Brief description of the Figures

Figure 1. Substrate specificity of elongase(s) from mid-developing nasturtium (*T. majus*) embryos. 200 μg of protein from a 15,000 *x g* particulate fraction was used in the elongase assay. Reaction conditions were as described in the Detailed Description of the Invention. Results represent the average of three replicates. For each [1-^{14}C]-acyl-CoA substrate, the relative proportional distribution of radiolabeled fatty acid elongation product(s) is (are) demarcated.

Figure 2 A: Comparison of the amino acid sequences of the nasturtium FAE homolog (NasFAE; accession no. AY0826190) with fatty acid elongase1 (FAE1) and related 3-ketoacyl-CoA synthases from other plant species. The alignment contains the sequences of the corn (ZeaFAE), *Limnanthes* (LimFAE), jojoba (SimFAE), *Arabidopis* (AraFAE) *Brassica* (BraFAE) and two *Arabidopis* 3-ketoacyl-CoA synthases associated with wax synthesis (AraKCS, AraCUT). The GenBank Accession numbers for the sequences shown are AJ292770 (ZeaFAE), AF247134 (LimFAE), U37088 (SimFAE), AF053345 (AraKCS), AF129511 (AraACUT), U29142 (AraFAE), AF009563 (BraFAE). Conserved cysteine and histidine residues are labeled with diamonds and triangles, respectively. Tyrosine at position 429 in the nasturtium FAE polypeptide is indicated by an asterisk. **B:** Dendrogram of the 3-ketoacyl-CoA synthase gene family based on the amino acid sequences. The alignment was carried out by the Clustal W method using Lasergene analysis software (DNAStar, Madison, WI) GenBank accession numbers: AF247134 (LimFAE), U37088 (SimFAE), AY082610 (NasFAE), AJ292770 (ZeaFAE), AF053345 (AraKCS), U29142 (AraFAE), AF009563 (BraFAE), AF129511 (AraCUT).

Figure 3. Hydropathy analysis of *T. majus* FAE. **A:** Hydropathy plot of FAE indicating the presence of several hydrophobic regions. **B:** Schematic representation of the putative transmembrane domains of *T. majus* FAE amino-acid sequence as predicted by TMAP analysis (Persson and Argos 1994). Numbers shown in the boxes correspond to the residues of each domain in FAE.

Figure 4. Northern and Southern analyses of *T. majus FAE*.

A: Northern analysis of *FAE* gene expression in *T. majus*. Total RNA was isolated from roots (RT), leaves (LF), petals (PL) and embryos (EO). **B:** Southern blot analysis of the *FAE* gene in *T. majus*. Genomic DNA was digested with restriction enzymes: *Eco*RI (lane 1), *Acc*I (lane 2), *Nco*I (lane 3) and *Hind*III (lane 4).

Figure 5. A. Proportions of 20:1 Δ11 and 22:1 Δ13 in seed oils from non-transformed *A. thaliana* ecotype Wassilewskija (WS-Con), two plasmid only transgenic control lines (RD1- and RD-15), and the eighteen best *A. thaliana* T_2 transgenic lines expressing the *T. majus FAE* gene

under control of the napin promoter. **B.** Proportions of 18:0, 20:0, 22:0 and 24:0 in seed oils from non-transformed *A. thaliana* ecotype Wassilewskija (WS-Con), two plasmid only transgenic control lines (RD1- and RD-15), and the eighteen best *A. thaliana* T_2 transgenic lines expressing the *T. majus FAE* gene under control of the napin promoter. The values are the average ± SD of three determinations performed on 200-seed lots.

Figure 6. The accumulation of erucic acid (22:1) in T_1 mature seeds of non-transformed *Brassica carinata* wild-type control (ntB, Black bar) and *Brassica carinata* transformed with the nasturtium *FAE* gene (NF Lines, Gray bars).

Figure 7. The accumulation of erucic acid (22:1) in T_1 mature seeds of non-transformed *Brassica carinata* wild type control (ntB, Black bar) and *Brassica carinata* transgenic lines carrying both the *Arabidopsis FAE1* and nasturtium *FAE* genes (Lines 6A through 33G ; Gray bars).

Figure 8. The accumulation of 20:1 Δ5 and 22:2 Δ5, Δ13 in T_2 mature seeds of *Arabidopsis thaliana*-ecotype Wassilewskija non-transformed wild-type (nt-WT) and empty vector only controls, and *Arabidopsis thaliana* of the same ecotype co-transformed with the nasturtium *FAE* and *Limnanthes* D5 desaturase genes (NFPC/D5 Lines).

Figure 9. Dendrogram of the 3- ketoacyl-CoA synthase gene family based on the amino acid sequences. The alignment contains the protein **sequence of the *Crambe abyssinica* FAE (CrFAE)**, compared with those of *Brassica juncea* FAE1 (BjFAE), *Brassica oleracea* FAE1 (BoFAE), Brassica *napus* FAE1 (BnFAE), *Arabidopsis thaliana* FAE1 (AtFAE) and *Tropaeolum majus* FAE (TmFAE).

Figure 10. Hydropathy analysis of *Crambe abyssinica* FAE. (**A**) Hydropathy plot of FAE indicating the presence of several hydrophobic regions. (**B**) Schematic representation of the putative transmembrane domains of *C. abyssinica* FAE amino-acid sequence as predicted by TMAP analysis [Persson, Argos 1994]. Numbers shown in the boxes correspond to the residues of each membrane domain in FAE.

Figure 11. Gas chromatogram of fatty acid methyl esters (FAMEs) extracted from yeast cells transformed with **A**: *Crambe abyssinica FAE* homolog in pYES2.1/V5-His-TOPO plasmid and **B**: empty pYES2.1/V5-His-TOPO (control).

Figure 12. The accumulation of erucic acid (22:1) in T_1 mature seeds of non-transformed *Brassica carinata* wild-type control (ntB, Black bar) and *Brassica carinata* transformed with the *Arabidopsis FAE1* gene (Lines 2B through 37B; Gray bars).

Figure 13. The accumulation of 22:1 and VLCFAs in T_2 mature seed of *B. napus* cv. Hero non-transformed wild-type controls (H-WT) and eight Hero/*FAE1* transgenic lines (H-10-1 to H-20-6). Fatty acid proportions are shown as the % (w/w) of total fatty acids. Each bar represents the mean ± S.D. of ten single seed analyses.

Figure 14. Proportions of erucic acid, total very long chain fatty acids (VLCFA) and oil content in the best seven DH *B. napus* c.v. Hero/*FAE1* transgenic lines and c.v. Hero and elite c.v. Millennium wild-type control plants from field trials. The results represent average ±SD of twelve seed samples from ten plants for each transgenic DH line and wild-type controls (WT).

Detailed Description of the Invention

Example 1

Plant materials

All experimental lines propagated in the greenhouse were grown at the Kristjanson Biotechnology Complex greenhouses, Saskatoon, under natural light conditions supplemented with high-pressure sodium lamps with a 16 h photoperiod (16 h of light and 8 h of darkness) at 22°C and a relative humidity of 25 to 30%. *Tropaeolum majus* plants (cultivar Dwarf Cherry Rose) were grown in the greenhouse and flowers were hand-pollinated. Seeds at various stages of development were harvested, their seed-coats were removed and embryos were frozen in liquid nitrogen and stored at -80^{0}C. Tobacco plants were grown under sterile conditions on MS medium (Murashige and Skoog, 1962) as well as under normal greenhouse conditions. *Arabidopsis* plants were grown in a growth chamber at 22°C with photoperiod of 16 h light (120 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 8 h dark.

Nasturtium embryo protein preparations and elongase assays

Embryos (2-3 grams) were ground in a mortar under liquid nitrogen and then 10 ml of IB buffer (80 mM HEPES pH 7.2, containing 2 mM DTT, 320 mM sucrose and 5% PVPP) per g fresh weight was added. The homogenate was filtered through Miracloth and spun for 5 min at 5, 000 *x g* in a Sorvall refrigerated centrifuge at 5 °C, the supernatant retained and re-centrifuged at 15, 000 *x g* for 25 min. The resulting pellet was resuspended in 80 mM HEPES containing 20% glycerol and 2 mM DTT. The concentration of protein was determined by the BioRad micro-Bradford method. This subcellular fraction was either used directly to determine enzymatic activities or stored at -80^{0}C until used.

The 15,000 *x g* particulate preparation was used to perform elongation assays as described by Taylor et al., (1992a & b) with the following modifications: The assay mixture consisted of 80 mM HEPES-NaOH, pH 7.2 containing 0.75 mM ATP, 10 μM CoA-SH, 0.5 mM NADH, 0.5 mM NADPH, 2 mM $MgCl_2$, 200 μM malonyl-CoA, 18 μM [1-^{14}C] acyl-CoA (0.37 GBq · mol^{-1}) and nasturtium protein in a final volume of 500 μL. The reaction was started by the addition of 200 μg of protein and incubated in a shaking water-bath at 30°C, 100 rpm for 0.5 h. [1-^{14}C] - Radiolabeled acyl-CoAs were synthesized from the corresponding free fatty acids as described previously by Taylor et al., (1990). Elongase reaction assays were stopped with 3 mL of 100gL^{-1} KOH in methanol. Fatty acid methyl esters (FAMEs) were prepared and quantified by radio-HPLC as described previously (Taylor et al., 1992b).

Lipid analyses

The total fatty acid content and acyl composition of tobacco plant lipids and *Arabidopsis* seed oils was determined by GC of the FAMEs with 17:0 FAME as an internal standard as described previously (Zou et al., 1997; Katavic et al., 2001; Taylor et al., 2001)

Isolation of nasturtium *FAE* cDNA by a degenerate primers approach

Degenerate primers were designed for amino acid sequences conserved among *Arabidopsis thaliana* KCS1 (AF053345), *Brassica napus* FAE1 (AF009563), *Limnanthes douglasii* FAE (AF247134) and *Simmondsia chinensis* FAE (U37088). Single-stranded cDNA template for reverse transcriptase-PCR was synthesized at 42°C from embryo poly (A) RNA with PowerScript™ (Clontech). A 50 µL PCR reaction contained single-stranded cDNA derived from 40 ng of poly (A) RNA, 20 pM of each primer: F1 -forward TCT(A/T)GG(A/T)GG(C/A)ATGGGTTG (SEQ ID NO:1) [LGGMGC] (SEQ ID NO:2), R1-reverse T(G/A)TA(T/C)GC(C/T)A(A/G)CTC(A/G)TACC (SEQ ID NO:3) [WYELAY] (SEQ ID NO:4) and 2.5 U of Taq DNA Polymerase (Amersham) under standard conditions. An internal part of the elongase sequence was amplified in a thermocycler during 30 cycles of the following program: 94°C for 30 sec, 48°C for 30 sec and 72°C for 1 min. The sequence of a 650-bp PCR product was used to design a primer to amplify the 5' and 3' ends of the cDNA using the SMART™ RACE cDNA Amplification Kit (CLONTECH). After assembly to determine the full length sequence of the cDNA, the open reading frame (ORF) was amplified using the primers P-forward ACCATGTCAGGAACAAAAGC (SEQ ID NO:5) and PR-reverse TTAATTTAATGGAACCTCAACCG (SEQ ID NO:6), and subsequently cloned into the pYES2 expression vector (Invitrogen).

cDNA library construction

To construct the nasturtium developing cDNA library, immature seeds were collected 17 days after pollination. Total RNA was extracted from embryos according to Lindstrom and Vodkin (1991), then poly (A) RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (DYNAL). Copy DNA synthesis was performed on 1 µg of poly (A) RNA using SMART PCR cDNA Synthesis Kit (Clontech) according to manufacturer's protocol. The cDNA population was then subtracted with 12S and 2S seed storage protein cDNA clones using PCR-Select cDNA Subtraction Kit (Clontech). The subtracted embryo cDNA population was cloned and then sequenced as described by Jako et al. (2002).

Sequence handling

Sequence analyses were performed using Lasergene software (DNAStar). Sequence similarity searches and other analyses were performed using BLASTN, BLASTX and PSORT programs.

Site directed mutagenesis of FAE

A site-directed mutagenesis experiment was performed essentially as described previously (Katavic et al., 2002). The desired mutation (tyrosine at position 429 is replaced with histidine) was introduced into the FAE coding region by polymerase chain reaction using primers F2-forward TCGAGGATGTCGCTTCACCGATTTGGAAACAC (SEQ ID NO:7) and R2-reverse GTTTCCAAATCGGTGAAGCGACATCCTCGATGG (SEQ ID NO:8). Primers were complementary to the opposite strands of pYES2.1/V5-His-TOPO containing the nasturtium *FAE* gene.

Northern analysis

Total RNA from nasturtium plant material was isolated according to Lindstrom and Vodkin (1991). 20 microgram of RNA was fractionated on a 1.4% formaldehyde-agarose gel and the gels were then stained with ethidium bromide to ensure that all lanes had been loaded equally. The RNA was subsequently transferred to Hybond N^+ membrane and hybridized with the ^{32}P labeled *FAE* DNA probe, prepared using the Random Primers DNA labeling kit (Gibco-BRL, Cleveland). Membranes were hybridized at 60°C overnight.

Plant transformation vectors

The coding regions of the nasturtium *FAE* (natural and mutated versions named SF and SMF, respectively) were amplified by polymerase chain reaction with primers F3-forward: taggatccATGTCAGGAACAAAAGC (lower case indicates the restriction site for BamHI) (SEQ ID NO:9); and R3-reverse tagagctcTTAATTTAATGGAACCTCAACC (lower case indicates the restriction site for *Sac*I enzyme) (SEQ ID NO:10) and subsequently cloned as a *Bam*HI and *Sac*I fragment behind the constitutive 35S promoter in binary vector pBI121 (CLONTECH).

The coding region of the nasturtium *FAE* was cloned behind the seed-specific napin promoter as follows: A *Bam*HI site was introduced in front of the start codon and behind the stop codon of *FAE* by PCR with primers F3 (as above) and R4-reverse: taggatccTTAATTTAATGGAACCTCAACC (lower case indicates the restriction site for *Bam*HI) (SEQ ID NO:11). The *B. napus* napin promoter was cloned in *Hind*III/*Xba*I sites of the pUC19 (Fermentas) and the nos terminator was introduced as an *Eco*RI/*Bam*HI fragment. The resulting vector was named pDH1. The napin promoter/nos terminator cassette was excised by *Hind*III/*Eco*RI digestion and subsequently cloned into the respective sites of pRD400 (Clontech) resulting in pVK1. The coding region of *FAE* was then cloned into the *Bam*HI site of pVK1 behind the napin promoter and the resulting vector was named NF. Sense orientation of the *FAE* coding region with respect to the promoter was confirmed by restriction analyses with *Xba*I.

The final binary vectors (SF: 35S-FAE, SMF: 35S-Mutated FAE, and NF: napin: FAE) were electroporated into *Agrobacterium tumefaciens* cells strain GV3101 containing helper

plasmid pMP90. Plasmid integrity was verified by DNA sequencing following its re-isolation from *A. tumefaciens* and transformation into *E. coli*.

Plant transformation and genetic analysis

Tobacco (*Nicotiana tabacum* cv. Xanthi) was transformed using a leaf disc transformation procedure (Horsch et al., 1985). Shoots that rooted in the presence of 50 µg/mL kanamycin were considered to be transgenic. Transgenic plants were transferred to soil and grown in the greenhouse.

Arabidopis (*A. thaliana* ecotype Wassilewskija) were transformed by vacuum infiltration according to the method of Clough and Bent (1998). Transgenic plants were selected and analyzed essentially as described by Jako et al., (2001).

Molecular analysis of transgenic plants

DNA was isolated from 2-3 g of tobacco or 150 mg of *Arabidopsis* leaf material using a urea-phenol extraction method (Chen et al., 1992) with the following minor modification: Material was frozen in liquid nitrogen and kept at -80^{0}C until used. Extraction was performed for 15 min at room temperature and 400 mM ammonium acetate, pH 5.2 was used for the first two precipitation steps. Stable integration of the napin:*FAE*:nos cassette into the genome of transgenic plants was checked by PCR amplification on genomic DNA with NN3 and NN4 primers as described by Katavic et al., (2001).

Southern analyses were performed to further confirm and select those transformants containing single or multiple copies of the inserted fragments. 15 microgram of tobacco or 1 microgram of *Arabidopsis* genomic DNA was digested with the restriction enzyme *Sac*I, and the resulting fragments were separated on a 0.9% (w/v) agarose gel, transferred to Hybond N$^+$ nylon membrane (Amersham) via an alkali blotting protocol. A 1.5 Kbp probe containing the coding sequence of *FAE* was generated by polymerase chain reaction (PCR) using primers: F4-forward ATGTCAGGAACAAAAGC (SEQ ID NO:12) and R5-reverse TAATTTAATGGAACCTCAACCG (SEQ ID NO:13) and subsequently radioactively labeled with ^{32}P as described above. Hybridization was performed at 65°C. The filters were washed once in 1x SSPE, 0.1% SDS for 15 min and in 0.1x SSPE, 0.1% SDS for 5-10 min at the temperature of hybridization. The blots were developed by exposure to X-OMAT-AR film (Kodak, Rochester, NY).

To estimate the number of *FAE* isoforms in the *T. majus* genome, 15 microgram of genomic DNA was digested with restriction enzymes: *Eco*RI, *Acc*I, *Nco*I and *Hind*III. Blotting and hybridization conditions were essentially as above except that filters were washed at low stringency with 1x SSPE, 0.1% SDS for 15 min, autoradiographed and then washed subsequently with 0.1x SSPE, 0.1% SDS, and re-exposed.

Example 2

Acyl Composition of *T. majus* cv Dwarf Cherry Rose

The acyl composition of the TAG fraction of this cultivar was typical in that it had highly enriched proportions of very long chain monounsaturated fatty acids (VLCMFAs), particularly 22:1 (77.5%) and 20:1 (16.0%) with a trace of 24:1 (1.5%), and a low proportion of total C_{18} fatty acids (2.5%), primarily 18:1 (2.4%). The predominant TAG species were trierucin followed by 22:1/20:1/22:1 (Taylor et al., 1992a).

Example 3

Substrate specificity of nasturtium embryo elongases *in vitro*

Although there has been considerable debate regarding the acyl substrate for elongase activity in developing oilseeds, recent studies of developing seeds of *B. napus* have revealed the presence of two types of elongation activity *in vitro*: an acyl-CoA-dependent activity, and an ATP-dependent activity which apparently utilizes an endogenous acyl primer. A 15,000 *x g* particulate fraction was isolated from nasturtium embryos collected at mid-development (at 14-17 days after pollination), the stage which exhibited the highest enrichment in acyl-CoA-dependent elongase activity.

It has been shown that while ATP is necessary for acyl-CoA-dependent elongation *in vitro*, too high a concentration of ATP strongly inhibited elongase activity. In addition, elongase enzyme activity has been reported to be stimulated by the presence of 10 μM CoASH. In order to optimize reaction conditions, we assessed the roles of these two co-factors. Elongase activity was measured *in vitro* in the 15,000 *x g* particulate fraction from nasturtium embryos under different ATP concentrations (0-5 mM) in the presence of 10 μM CoASH with 18 μM 1-[^{14}C]-18:1-CoA and 200 μM malonyl-CoA. The highest activity was found at a concentration of 0.75mM of ATP. Then, elongase activity was examined with range of [1-^{14}C]-acyl-CoAs substrates at an ATP concentration of 0.75mM in the presence of 10 μM CoASH.

Our results indicate that in a developing nasturtium embryo particulate fraction, acyl-CoA-dependent elongases have the capacity to elongate a wide range of saturated (C_{16}-C_{20}) and monounsaturated (C_{18} and C_{20}) fatty acyl moieties (Fig. 1). Of the [1-^{14} C]-labeled acyl-CoA series (16:0-CoA, 18:0-CoA, 18:1-CoA, 20:0-CoA, 20:1-CoA, 22:1-CoA), tested *in vitro*, elongase(s) from mid-developing nasturtium embryos exhibited the highest activity with 18:1-CoA and 20:1-CoA (102 and 95 pmol/min/mg protein, respectively). These elongase activity rates are of the same order of magnitude as that reported for acyl-CoA elongase(s) in a similar particulate fraction from developing rapeseed embryos. The particulate fraction was also able to elongate, in order of specificity, the saturated substrates 18:0-CoA, 16:0-CoA, and to a much lesser extent, 20:0-CoA. In general, regardless of the 1-[^{14}C]acyl-CoA substrate supplied *in vitro*, the major labeled fatty acyl product was the C_2 extension of its respective precursor (about 80-90%), with the next

respective C_4 extension product being present in proportions of about 10-20% (Figure 1). The one critical exception to this trend was the production solely of radiolabeled erucic acid from its respective 1-[^{14}C] eicosenoyl-CoA precursor. There was no detectable elongation of 1-[^{14}C]-labeled 22:1-CoA to 24:1, even though the latter is found in trace amounts in nasturtium seed oil.

Example 4

Isolation of *T. majus FAE* homolog

Based on sequence homology among plant fatty acid elongase genes, a full-length clone was amplified by PCR using a degenerate primers approach and the sequence submitted to the GenBank (accession number AY082610; Figure 2 (A)). The nucleotide sequence had an open reading frame of 1512 bp. Subsequently, 3 partial clones of about 0.6 kb, representative of the AY083610 *FAE* clone, were found among 2,800 ESTs surveyed (about 0.1% representation) from a nasturtium embryo subtracted cDNA library.

Alignment of the amino acid sequence of the nasturtium FAE with other plant condensing enzymes revealed the presence of six conserved cysteine residues (Fig 2A.). Further sequence analysis showed that one out of the four conserved histidine residues suggested to be important for *Arabidopsis* FAE1 activity, was substituted with tyrosine in the *T. majus* FAE polypeptide.

An analysis of the nucleotide sequence of the corresponding nasturtium *FAE* genomic clone revealed the absence of intron sequences. A similar absence of introns was observed in homologs from

A. thaliana FAE1, rapeseed CE7 and CE8 and high and low erucic lines of *B. oleracea, B. rapa*, canola *B. napus* cv Westar and HEAR *B. napus* cv Hero.

The *T. majus* FAE cDNA encodes a polypeptide of 504 amino acids that is most closely related to an FAE2 from roots of *Zea mays* (69 % amino acid identity) (Fig. 2 (B)). The *T. majus* FAE polypeptide also shared strong identity with FAEs from *Limnanthes douglasii* (67%) and from seeds of jojoba (*Simondsia chinensis*) (63%). Homology of the nasturtium FAE to two *Arabidopsis* 3-ketoacyl-CoA synthases AraKCS and AraCUT1) involved in cuticular wax synthesis was on the level of 57% and 53%, respectively. These homologs all exhibit the capability to elongate saturated fatty acids to produce saturated VLCFAs. The FAE1 polypeptides involved in the synthesis of VLCFAs in *Arabidopsis* and *Brassica* seeds showed approximately 52-54% identity with the *T. majus* FAE. The nasturtium FAE protein was predicted to have a theoretical pI value of 9.3 using the algorithm of Bjellqvist et al., (1993 and 1994) and a molecular mass of 56.8 kDA, which are similar to the respective values reported for the *B. napus* CE7 and CE8 FAE homologs as well as those from *B. rapa* (*campestris*) and *B. oleracea*.

A hydropathy analysis (Kyte-Doolittle) of the amino acid sequence of the *T. majus* FAE revealed several hydrophobic domains (Fig. 3A). Protein analyses with the TMAP algorithm (Person and Argos, 1994) predicted two *N*-terminal transmembrane domains, the first

corresponding to amino-acid residues 35-55, and the second spanning residues 68-88 (Fig 3B). Like other elongase condensing enzymes, the *T. majus* FAE lacks *N*-terminal signal sequences typically found for plastidial or mitochondrial-targeted plant enzymes. It also lacks a **KXKXX** or **KKXXX** motif (X=any amino acid) often found at the *C*-terminus of proteins retained within ER membranes. Rather, it is a type IIIa protein, typically present on endoplasmic reticular membranes.

Example 5

Tissue specific expression and copy number estimate of *T. majus FAE*

Northern blot analyses were performed to investigate the expression profile of the *FAE* gene. Total RNA was isolated from different nasturtium tissues including roots, leaves, floral petals and mid-developing embryos. A strong hybridization signal with *FAE*-specific probe was observed only with RNA isolated from developing embryos (Fig. 4. A).

A Southern blot hybridization was performed with nasturtium genomic DNA digested with several restriction enzymes including *Eco*RI, *Acc*I, *Nco*I and *Hind*III. The *FAE* gene has no internal *Eco*RI, *Acc*I or *Nco*I sites, while one internal *Hind*III site exists. Autoradiography revealed the presence of one strongly-hybridizing fragment in all cases except with *Hind*III for which two strongly hybridizing fragments were evident (Fig. 4.B). In addition a minimum of 4 weakly hybridizing fragments were detected. After washing under high stringency conditions, the number of hybridizing fragments was unchanged. Thus, we have concluded that *T. majus FAE* belongs to a multigenic family consisting of 4 to 6 members. A similar multigenic family has been found for a rapeseed *FAE1* gene member.

Example 6

Heterologous expression of the *T majus* FAE in Yeast

To study the function of the protein encoded by the *T. majus* FAE, the coding region was linked to the galactose-inducible *GAL1* promoter in the expression vector pYES2 and transformed into yeast. Transgenic yeast cells harbouring the *T. majus* FAE did not show any difference in fatty acid composition in comparison to yeast cells transformed with empty vector. A similar difficulty with expression of *Limnanthes* FAE and corn FAE in yeast cells has been reported.

As indicated earlier, a comparison of the predicted amino acid sequence of the nasturtium FAE with other plant condensing enzymes (Fig 2A) showed that one of the four conserved histidine residues, known suggested to be important for *Arabidopsis* FAE1 activity, was substituted with tyrosine in the *T. majus* FAE polypeptide. To study the importance of this histidine residue for enzyme activity, we used a site directed mutagenesis approach to replace the tyrosine 429 residue with histidine. This mutated version of nasturtium FAE was expressed in yeast cells. Analyses of fatty acid composition of transformed yeast cells showed that histidine at position 429 did not restore enzyme activity. Therefore we decided to study the function of *T. majus* FAE in plant heterologous chromosomal backgrounds.

Example 7

Expression of *T. majus FAE* in tobacco plants

To establish functional identity, the cDNA for the FAE-related polypeptides was constitutively expressed in tobacco plants under the control of the tandem 35S constitutive promoter. In addition, to assess the importance of histidine residues for enzyme activity, the tyrosine at position 429 in the nasturtium FAE was replaced with histidine and subsequently used to prepare a plant transformation vector under the control of the tandem 35S promoter. Integration of the 35S/*FAE*/Nos expression cassette into tobacco plants was confirmed by PCR amplification on genomic DNA. Fatty acid composition was determined in callus, leaves and seeds of transgenic tobacco plants.

Constitutive expression of the nasturtium FAE homologue in tobacco callus resulted in an increase in proportions of VLCFAs from 3.7 % in the wild type background to as high as 8.6% (a 132% increase) in transgenic lines (Table I). In particular, the increase in proportions of saturated VLCFAs (22:0, 24:0, and 26:0) was most pronounced. The fact that the synthesis of the saturated VLCFAs occurs at the expense of 16:0 and 18:0 suggests that the nasturtium FAE is able to elongate C_{16} and C_{18} fatty acids. Expression of the mutated version of the nasturtium FAE (SMF) resulted in a slight increase in the VLCFA content in tobacco callus, on average 18.5% in comparison to the wild type background. Increased proportions of VLCFAs at the expense of LCFAs were observed in leaves of transgenic tobacco plants carrying either the nasturtium FAE or its mutated form (Table II). Comparison of fatty acid composition in tobacco tissues upon expression of the nasturtium FAE and its mutated version, revealed that tyrosine at position 429 is likely important to achieve full activity of the enzyme. A decreased proportion of 18:3 in leaves of transgenic tobacco lines in comparison to the wild type (empty vector) background suggests that the "metabolic pull" of the elongation pathway may be somewhat stronger than that of the competing desaturation pathway.

Expression of nasturtium *FAE* in tobacco seeds resulted in a 50% increase in proportions of VLCFAs from 0.6% in the wild type background to 0.9% in transgenic plants (data not shown). The relatively low proportions of VLCFAs in tobacco leaves and callus (see Tables I and II) may be an indication that (i) *in vivo*, saturated fatty acids are not present at high concentrations; therefore even a 50% increase in relative proportions does not result in high levels of VLC saturated fatty acids accumulating in glycerolipids; (ii) expression of the nasturtium *FAE* when under the control of the 35S promoter is relatively weak.

Example 8

Expression of *T. majus FAE* in *Arabidopsis* seeds

Since expression of nasturtium *FAE* under the control of the 35S promoter did not result in a high accumulation of VLCFA in tobacco seeds we decided to study the effect of expressing it in *Arabidopsis*. Using a vacuum-infiltration method, 18 kanamycin resistant *Arabidopsis* plants were obtained. The fatty acid composition of T_2 seeds was determined. A significant increase was observed only in the content of erucic acid (22:1 c13). On average, the level of erucic acid increased up to 3.2% (a 50% increase) in transgenic seeds comparing to 2.1% in wild type background (data not shown). In the best transgenic lines, the content of erucic acid increased up to 4.0% (a 90% increase).

Since tandem 35S-driven constitutive *FAE* expression did not result in a strong increase in VLCFA proportions in tobacco and *Arabidopsis* seeds, we decided to use the seed-specific promoter napin to study *FAE* expression in an *Arabidopsis* seed background. From vacuum-infiltration experiments, 25 kanamycin-resistant T_1 plants were selected. The T_2 progeny were collected individually from each plant and the fatty acid composition determined. Significant changes in fatty acid composition in comparison to the wild type (empty vector) were found. On average, the proportion of erucic acid (22:1 Δ13) increased from 2.1% in wild type to 9.6% in T_2 transgenic seeds at the expense of 20:1 Δ11 (Table III). Eighteen of the best transgenic lines were selected to examine the range of VLCFA proportional re-distribution induced by expression of the nasturtium FAE gene (Figure 5A and B). The erucic acid content was increased by up to 6.5-fold in line NF-8. Small increases in the proportions of 24:1 Δc15 were also observed (Table III). There was also a relatively significant increase in the proportions of the saturated VLCFAs, 22:0 and 24:0, at the expense of 18:0 and 20:0. In both the case of the VLC mono-unsaturated fatty acids (Fig 5A) and the VLC saturated fatty acids (Fig 5B), the highest proportional increases in erucic and in [behenic + lignoceric] acids were generally correlated with the concomitant reduction in the proportion of their corresponding elongase primers, eicosenoic and [stearate + arachidic] acids, respectively.

Therefore, we conclude that the nasturtium FAE is able to preferentially elongate 20:1 and [18:0 + 20:0]. As would be expected, there was significant variation in the proportions of 22:1 which accumulated (Figure 5A) possibly due to positional effects from nasturtium *FAE* transgene insertion at different sites in the *Arabidopsis* genome. Similar variations were observed in the expression of a castor fatty acid hydroxylase gene (*CFAH12*) in the *Arabidopsis fad2/fae1* mutant.

In summary, we have isolated a cDNA clone from nasturtium which exhibits high similarity to the sequences of 3-ketoacyl-CoA synthases from various plant species but has the unprecedented capability to increase the erucic acid content by 8-fold in *Arabidopsis thaliana*.

Our *in vitro* findings suggest that the FAE proteins in a 15,000*x g* nasturtium particulate fraction have a broad acyl-CoA preference, with the ability to elongate both monounsaturated and saturated C_{18}-CoA and C_{20}-CoA substrates. In like manner, a partially purified jojoba FAE1 showed maximal activity with monounsaturated and saturated C_{18} and C_{20}-CoAs *in vitro*. However, it is important to note that the particulate elongation activity reported in the current study most likely represents the cumulative effect of expression of more than one member of this small gene family. Thus, from this experiment one can only conclude that the capacity to elongate both monounsaturated and saturated acyl moieties is represented in this nasturtium particulate fraction.

While genetic analyses and homology assessments might predict that the isolated nasturtium *FAE* gene might encode an enzyme which prefers to elongate saturated acyl-CoAs, the transgenic experiments in tobacco callus, tobacco leaves and in *Arabidopsis* seed, collectively confirmed that the heterologously-expressed *T. majus FAE* gene product can elongate both monounsaturated and saturated acyl moieties. In fact, in a transgenic *Arabidopsis* background, the nasturtium FAE was much stronger than the jojoba 3-KCS in its ability to increase the level of 22:1: Introducing the jojoba cDNA into *Arabidopsis* resulted in an increase in 22:1 proportions from about 2% in the control to 4% in the transgenics. In comparison, when we introduced the *T. majus* FAE into *Arabidopsis*, the erucic acid content increased by almost an order of magnitude (8-fold) at the concomitant expense of 20:1 Δ11. The acyl composition of the transgenic *Arabidopsis* seed oil was reproportioned such that erucic and eicosenoic became about equal as the two predominant VLCFAs.

The ability of the nasturtium FAE protein to preferentially elongate 18:1-CoA and especially 20:1-CoA, is consistent with the observed acyl composition of nasturtium seed oil which consists primarily of very long chain- and specifically erucoyl moieties. We postulate therefore, that whether the nasturtium *FAE* transgene results in predominantly mono-unsaturated (20:1 Δ11, 22:1 Δ13) or saturated (e.g. 20:0, 22:0) VLCFAs is more a function of the composition of the acyl-CoA pool (18:1 Δ9 and 20:1 Δ11 or 18:0 and 20:0 or, respectively) available to the condensing enzyme in the host species/target organ.

Thus, the nasturtium *FAE* homolog described herein, will have a larger engineering impact when strongly expressed in a seed-specific manner in H.E.A. *Brassicaceae* (e.g. *B. napus; B. carinata*) wherein 18:1 Δ9 [and 18:2/18:3] and 20:1 Δ11 represent a potential acyl-CoA elongation substrate pool of almost 40% to enrich the already-existing 22:1 Δ13 content. Clearly, the current studies indicate that the nasturtium *FAE* expression should be combined with other genetic modifications we have made to enhance the VLCFA content of HEAR *Brassicaceae* and the proportions of erucic acid in particular, to provide an industrial feedstock oil of high value and broad applicability.

A major goal of our research is to obtain, by genetic manipulation, *Brassica napus* L. cultivars or *B. carinata* breeding lines with higher levels of erucic acid (22:1) in their seed oil than in present Canadian HEA cultivars. For example, we would like to develop a *B. napus* cultivar containing erucic acid levels above 66 mol%, ideally with more than 80% erucic acid in the seed oil. To achieve our goals we are isolating new, more efficient strategic genes for high erucic acid and preferably, trierucin, production. We selected *Tropaeolum majus*, garden nasturtium, as a source of those genes based on the fact that this plant is capable of producing significant amounts of erucic acid (70-75 % of total fatty acid) and accumulates trierucin as the predominant TAG in its seed oil. The fatty acid elongase (FAE), 3-ketoacyl-CoA synthase (KCS) is the first component of the elongation complex involved in synthesis of erucic acid (22:1) in seeds of *Tropaeolum majus* (garden nasturtium). Using a degenerate primers approach, a cDNA of an embryo *FAE* was obtained and heterologously expressed in two different plant backgrounds (*A. thaliana and N. tabacum*) under the control of a seed-specific (napin) promoter and the constitutive (tandem 35S) promoter, respectively. Seed-specific expression resulted in up to an 8-fold increase in erucic acid proportions in *Arabidopsis* seed oil. Constitutive expression in transgenic tobacco tissue resulted in increased proportions of very long chain saturated fatty acids. These results indicate that the nasturtium *FAE* gene encodes a condensing enzyme involved in the biosynthesis of very-long-chain fatty acids, utilizing monounsaturated and saturated acyl substrates. Thus, the nasturtium *FAE* homolog will have a larger engineering impact when strongly expressed in a seed-specific manner in H.E.A. *Brassicaceae* (e.g. *B. napus; B. carinata*) wherein 18:1 Δ9 [and 18:2/18:3] and 20:1 Δ11 represent a potential acyl-CoA elongation substrate pool of almost 40% over and above the existing high 22:1 Δ13 content..

In addition, heterologous expression of the nasturtium *FAE* gene in HEAR *Brassicaceae* can be combined with other genetic modifications we have made to enhance the VLCFA content of HEAR germplasm (Katavic et al., 2001; Taylor et al., 2001) and the proportions of erucic acid in particular, to provide an industrial feedstock oil of high value and broad applicability.

Expression of nasturtium *FAE* in *Arabidopsis* seeds resulted in an 8-fold increase in erucic acid content. Therefore, it is anticipated that the introduction of this gene alone, or in combination with other altered gene expression phenotypes (e.g. *FAE1* and/or *FAD2* and/or *FAD3*) into HEAR *Brassicaceae* will result in transgenic lines with improved proportions of erucic acid in the seed oil.

Example 9

Heterologous Expression of the nasturtium *FAE* in HEAR *Brassicaceae*- e.g. *B. carinata*

The nasturtium *FAE* gene under the control of the strong seed-specific promoter napin, was introduced into HEAR *Brassicaceae* (e.g. *B. carinata*). Considering the results obtained in

Arabidopsis seeds, we anticipated that there would be a strong increase in the proportion of 22:1 and saturated VLCFAs as well (by up to 8-10%).

The coding region of nasturtium *FAE* was cloned behind a napin promoter as described in Example 1. The resulting plasmid named NF was electroporated into *Agrobacterium tumefaciens* and subsequently used to transformed *Brassica carinata* plants using the protocol described by Babic et. al., (1998). Shoots that rooted in the presence of 25mg/L kanamycin were considered to be transgenic. Transgenic plants were transferred to soil and grown in the growth chamber. T_1 seed from self pollinated plants were harvested and subjected to biochemical analysis performed as described by Mietkiewska et al (2004). The proportion of erucic acid increased from 30% in wild type controls to as high as 39% in T_1 segregating seeds of the best transgenic line (Figure 6).

Example 10:

Heterologous co-expression of two *FAE* genes in HEAR *Brassicaceae* (e.g. *B. carinata*) co-transformed with the napin:*AthalFAE1*+ napin:*NastFAE*

Expression of nasturtium *FAE* in HEAR *Brassicaceae* (e.g. *B. carinata*) and the resulting proportional increase in erucic acid can be maximized by also addressing the fact that 20:1, the preferred monounsaturated substrate, is present in wild type seeds in relatively low proportions (5.5-6.5%). Therefore, for example, one can introduce the *Arabidopsis FAE1* and nasturtium *FAE* into HEAR *Brassicaceae* (e.g. *B. carinata*). The first gene product should enhance conversion of 18:1 to 20:1 (Katavic et al., 2001), while the nasturtium *FAE* gene product clearly prefers to extend 20:1 to 22:1. In this manner, the maximal proportion of erucic acid is expected. To achieve this goal, one could apply a co-transformation method: The *Arabidopsis FAE* is cloned in a derivative of vector pRD400 which allows selection on kanamycin, while the nasturtium *FAE* is cloned in pCAMBIA vector which allows selection on hygromycin.

The coding region of the nasturtium *FAE* with the nos terminator were excised from the SF plasmid (Mietkiewska et al., 2004) by *Bam*HI/*Eco*RI digestion. The napin promoter was excised from the NF plasmid (Mietkiewska et al., 2004) by a *HindIII*/*Bam*HI restriction reaction. Isolated fragments were cloned in *HindIII/EcoRI* sites of pCAMBIA1390 and the resulting plasmid was named NFPC. The binary vector (AFAE) containing the *Arabidopsis FAE1* under the control of the napin promoter was kindly provided by Dr L.Kunst, University of British Columbia, Vancouver, BC Canada and was constructed as described by Katavic et al, 2000 and by Katavic et al., 2001.

The binary vectors (NFPC, AFAE) were electroporated into *Agrobacterium tumefaciens* and subsequently introduced into *Brassica carinata* plants in a co-transformation experiment. Double transformants were selected on media supplemented with both kanamycin (25 mg/L) and hygromycin (10 mg/L). The selected plants were grown in the growth chamber. T_1 seeds were collected and subjected to biochemical analysis. As shown in Fig.7, significant changes in the

content of erucic acid were found in the double transformants compared to the control. The proportion of erucic acid increased from 30% in wild type to as high as 44- 46% in T_1 segregating seeds of the best transgenic lines.

Example 11:

Heterologous co-expression of the napin:*NastFAE* and the *Limnanthes* Des 5 desaturase genes co-transformed into *Arabidopsis thaliana*

The coding region of nasturtium *FAE* behind the napin promoter in pCAMBIA1390 vector (NFPC) was cloned as described in Example no.10. The coding region of *Limnanthes* Δ5 desaturase was cloned behind the napin promoter as follows: The plant transformation vector pSE129A, already prepared from pRD400 plasmid (Datla et al., 1992), was obtained by introducing a *Hind*III/*Xba*I fragment containing the *B. napus* napin promoter and a *Kpn*I/*Eco*RI fragment containing the *Agrobacterium nos* terminator. The 1.0 kb open-reading frame of the *Limnanthes Des5* (*Limnanthes* Acyl-CoA Δ5 desaturase, GenBank Accession no AF247133) was amplified by PCR with primers designed to contain *Xba*I and *Kpn*I restriction sites and was ligated into *Xba*I/*Kpn*I -digested pSE129A in the sense orientation to give the binary vector *Limdes5*/pSE. The binary vectors NFPC and Limdes5/pSE were introduced into *Agrobacterium tumefaciens* cells strain GV3101 as described in Example no.10.was then introduced by electroporation into *Agrobacterium tumefaciens* strain GV3101 bearing the helper plasmid pMP90 for plant transformation.*Arabidopsis* plants (*A. thaliana* ecotype Wassilewskija) were co-transformed by the vacuum infiltration method (Clough and Bent, 1998) using equal volumes of the two recombinant *A. tumefaciens* suspensions. Transgenic plants were selected and analyzed as described by Mietkiewska et al., (2004). Seeds (T_1) obtained from co-transformed plants were selected on media supplemented with both kanamycin (50 mg/l) and hygromycin (20 mg/l). The selected plants were grown in the growth chamber. T_2 seeds were collected and subjected to biochemical analysis (See Fig. 8).

Example 12:

Cloning of *Crambe abyssinica* 3-ketoacyl-CoA synthase (*FAE*) and heterologous expression in yeast.

Based on *FAE1* sequences from *Arabidopsis thaliana* and *Brassica napus*, the forward primer F5 (5'-GCAATGACGTCCATTAACGTAAAG-3') (SEQ ID NO:14) and the reverse primer R6 (5'-TTAGGACCGACCGTTTTGGGC-3') (SEQ ID NO:15) were designed and used to isolate the *FAE* coding region from *Crambe abyssinica*. Genomic DNA isolated from leaves according to urea-phenol extraction method (Chen et al., 1985) was used as a template for PCR amplification with Vent DNA polymerase (New England Biolabs) in a thermocycler during 30 cycles of the following program: 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min. A 1.5-kB PCR product was cloned into pYES2.1/V5-His-TOPO expression vector and subsequently

sequenced. The *Crambe FAE* in pYES2.1/V5-His-TOPO was transformed into *Saccharomyces cerevisiae* strain Inv Sci (Invitrogen) using the *S.c.* EasyComp transformation kit (Invitrogen). Yeast cells transformed with pYES2.1/V5-His-TOPO plasmid only were used as a control. The transformants were selected and grown as described previously (Katavic et al., 2002; Mietkiewska et al., (2004). Fatty acid methyl esters (FAMEs) from yeast cultures were extracted and analyzed as described by Katavic et al., (2002).

Example 13:

***Crambe FAE* Sequence Handling**

Sequence analyses were performed using Lasergene software (DNAStar, Madison, WI, USA). Sequence similarity searches and other analyses were performed using BLASTN, BLASTX and PSORT programs.

Example 14:

Isolation of *Crambe abyssinica FAE* homolog

Based on the sequence homology among plant fatty acid elongase genes, a coding region of the *FAE* gene from *Crambe abyssinica* was isolated and the sequence submitted to GenBank (Accession no. AY793549). The *Crambe FAE* open reading frame of 1521-bp encodes a polypeptide of 506 amino acid that is most closely related to an FAE1 from *Brassicaceae* (Fig. 9): *B. juncea* (97% identity, GenBank # AJ558198), *B. oleracea* (96% identity, GenBank # AF490460), *B. napus* (96% identity, GenBank #AF490459) and *B. rapa* (96% identity, GenBank #AF49041). The *Arabidopsis* FAE1 (GenBank # U29142) polypeptide showed 84% identity with the *Crambe* FAE. Previously isolated *Tropaeolum majus* FAE (GenBank #AY082610) showed 54% identity with the *Crambe* FAE. The *Crambe* FAE protein was predicted to have a molecular mass of 56.4 kD and a theoretical pI value of 9.29.

A hydropathy analysis (Kyte-Doolittle) of the amino acid sequence of the *Crambe* FAE revealed several hydrophobic domains (Fig 10A). Protein analyses with the TMAP algorithm (Persson and Agros, 1994) predicted two *N*-terminal transmembrane domains, the first corresponding to amino acid residues 9 to 31 and the second domain spanning residues 51 to 73 (Fig 10B).

Example 15:

Functional Heterologous expression of the *Crambe abyssinica FAE* in yeast cells

To study the function of the protein encoded by the *Crambe FAE*, the coding region was linked to the *GAL1*-inducible promoter in the yeast expression vector pYES2.1/V5-His-TOPO and transformed into *S. cerevisiae* strain InvSci1 yeast cells. As shown in Figure 11, yeast cells transformed with the plasmid containing the *Crambe FAE* open reading frame were found to have an accumulation of 20:1c11, 20:1c13, 22:1c13, 22:1c15 and 26:1c19; these are not present in wild-type yeast cells. In particular, the capability of the Crambe FAE to synthesize 20:1 c11 and 22:1

c13 are directly of interest for our target oil compositional changes as outlined in the Background section.

Example 16:

Heterologous Expression of the *Crambe FAE* in *Arabidopsis* and in HEAR *Brassicaceae*- e.g. *B. napus*; *B. carinata*

The coding regions of the *Crambe FAE* was amplified by polymerase chain reaction with primers: F6-forward: 5′-tatctagaATGACGTCCATTAACGTAAAG -3′(lower case- restriction site for *Xba*I) (SEQ ID NO:16) and R7-reverse: 5′-atggtaccTTAGGACCGACCGTTTTGG -3′ (lower case shows restriction site for *Kpn*I enzyme) (SEQ ID NO:17) and subsequently cloned behind the napin promoter in respective sites of pSE vector (Jako et al., 2001).

The final binary vector (napin/crambe *FAE*) was electroporated into *Agrobacterium tumefaciens* cells strain GV3101 containing helper plasmid pMP90 (Koncz and Schell, 1986). Plasmid integrity was verified by DNA sequencing following its re-isolation from*A. tumefaciens* and transformation into *E. coli*.

The binary vector was used to transform *A. thaliana* plants by the vacuum infiltration method (Clough and Bent, 1998) and high erucic *Brassica napus* plants using the methods of DeBlock et al, (1989) and *B. carinata* plants using the method of Babic et al., (1998).

Example 17:

Heterologous expression of the *Arabidopsis thaliana FAE* in HEAR *Brassicaceae*- e.g. *B. napus* cv Hero and in *B.carinata*.

The results from Millar and Kunst (1997) demonstrated that the heterologous expression of the *FAE* gene alone is sufficient for the production of VLCFAs and that it is the condensing enzyme that determines the quantity and the chain length of VLCFA synthesized by the microsomal fatty acid elongation complex.

Plant Material

High erucic acid Brassica *napus* L. cultivar Hero (Scarth et al., 1991) was obtained from the Plant Science Department of the University of Manitoba (Winnipeg, Canada).*B. napus* canola cultivar Westar was obtained courtesy of G. Rakow (Agriculture and Agri-Food Canada Research Center, Saskatoon). All experimental control and transgenic *B. napus* lines were grown simultaneously in the Kristjanson Biotechnology Complex greenhouses (Saskatoon) under natural light conditions supplemented with high-pressure sodium lamps with a 16 h photoperiod (16 light and 8 h of darkness) at 22°C and a relative humidity of 25 to 30%.

B. napus cv. Hero *SLC1-1* transgenic lines containing a yeast *sn*-2 *lyso*-phosphatidic acid acyltransferase (LPAT; EC 2.3.1.51) were produced and characterized as described previously (Zou et al. 1997). PCR and Southern analyses of the transgenic lines selected for further

biochemical characterization and field testing showed that all of the lines contained a single *SLC1-1* insert.

Lipid Substrates, Chemicals and Biochemicals

[1-^{14}C] oleic acid (2.15 x 10^9 Bq · mmol^{-1}) was purchased from Amersham Canada, Ltd. (Oakville, ON) and [1-^{14}C]-labeled oleic acid was converted to the corresponding labeled oleoyl-CoA using the method described by Taylor et al. (1990). Specific activity was adjusted as required by diluting with authentic unlabeled standard. Unlabeled oleoyl-CoA, malonyl-CoA, ATP, CoA-SH, NADH, NADPH, sodium acetate and most other biochemicals were purchased from Sigma. The 15:0 and 17:0 standards were supplied by Supelco Canada, Ltd. (Oakville, ON). HPLC-grade solvents (Omni-Solv, BDH Chemicals, Toronto, ON) were used throughout these studies.

***FAE1* Transformation Vector**

Drs A. Millar and L. Kunst (from the Dept. of Botany, University of British Columbia, Canada) kindly provided the binary vector pNap:*FAE1*/NGKM (Fig. 1) containing the *Arabidopsis thaliana FAE1* coding region under the control of seed-specific, napin promoter. The binary vector was introduced by electroporation into the *Agrobacterium tumefaciens* strain GV3101 bearing helper plasmid pMP90 and used in transformation experiments.

Transformation of *Brassica napus* cv Hero with the *FAE1* gene

Cotyledonary-petioles were excised from five to seven-day-old seedlings of the canola cv. Westar, and used as explants in transformation experiments. The transformation was carried out according to the method developed by Moloney et al., (1989).

Hypocotyls were excised from five to seven-day-old seedlings of the HEAR cv. Hero, and cut into 5 to 7mm segments. The hypocotyl explants were transformed using the method developed by DeBlock et al., (1989).

Experimental wild-type control plants were regenerated *in vitro* from the cotyledonary petioles and/or hypocotyl explants. Control explants were not co-cultivated with *A. tumefaciens*. However, with this exception, control explants were subjected to all the other experimental procedures and conditions applied to explants that were co-cultivated with *Agrobacterium* (and from which transformed shoots were developed).

Control and transformed shoots were rooted *in vitro* on rooting medium without kanamycin or with 25 μg·mL^{-1} kanamycin, respectively. Plants with well-developed roots were transferred to soil and grown to maturity. Developing and mature seed from self-pollinated control and transgenic lines grown in the greenhouse, were harvested and subjected to molecular and biochemical analyses.

Molecular analyses of transgenic plants

All molecular analyses (plasmid preparation, polymerase chain reaction (PCR), restriction digestion, DNA gel blot analyses etc.) were performed by methods prescribed by Sambrook et al. (1989) or Ausubel et al. (1995).

PCR amplification of the partial expression cassette NAP/*FAE1*/NOS

To check for integration of the napin:*FAE1*:nos transgene construct into the genome of putative transgenic plants, leaf tissue from T_0 plants was collected and genomic DNA isolated. This DNA was used as a template to amplify the partial expression cassette NAP/*FAE1*/NOS using oligonucleotide primer NN-3 (5'-TTTCTTCGCCACTTGTCACTCC-3') (SEQ ID NO:18) which was designed according to the promoter region of the napin gene (position 948-969) and primer NN-4 (5'-CGCGCTATATTTTGTTTTCTA-3') (SEQ ID NO:19) which was designed according to the nopaline-synthase 3' UTR sequence (position 1753-1773). The total size of the expected PCR product is *ca.* 2.0 Kb (0.197 Kb of napin promoter region + 1.608 Kb *FAE1* coding region + 0.204 Kb of nopaline-synthase 3' UTR region).

Seed Lipid and Protein Analyses

The total fatty acid content and acyl composition of seed lipids was determined by GC of the FAMEs (Fatty Acid Methyl Esters) with either 15:0 or 17:0 free fatty acid added as an internal standard, as described previously (Zou et al, 1997).

For analyses of the *FAE1* transgenic progeny, single seeds were cut with a scalpel into small pieces and an internal standard (15:0 free fatty acid) and 1 mL of 3 M methanolic-HCl (Supelco Canada, Ltd.) were added. Transmethylation was performed at 80°C for 2 h. Reaction mixtures were cooled on ice and 2 mL of 9 g L^{-1} NaCl was added. The mixture was extracted three times with 2 mL of hexane and then the hexane extracts were combined and taken to dryness under nitrogen. The acyl composition was determined by GC of the FAMEs on a Hewlett-Packard model 5890 gas chromatograph fitted with a DB-23 column (30m x 0.25 mm; film thickness, 0.25 μm; J & W Scientific, Folsom, CA). The GC conditions were: injector temperature and flame ionization detector temperature, 250°C; running temperature program, 180°C for 1 min, then increasing at 4°C/min to 240°C and holding this temperature for 10 min. Data from 10 single seed runs of each *FAE1* transgenic line were averaged.

For the *SLC1-1* and *FAE1* field trial progeny, a Near-Infrared Reflectance (NIR) method was used to estimate oil and protein content based on AOCS Procedure Am 1-92 (Firestone, 1998) using the NIR System 6500 (Foss North America), with software packages NEWISI and WINISI (Infrasoft International LLC). The sample size for NIR scanning was about 4.5 g, enough to fill the ring cup. The oil and protein contents as determined by NIR were calibrated against data obtained from NMR and Leco Protein Analyzer/Kjeldahl analyses (performed with a standard set of HEAR seed samples) (Tkachuk,1981), respectively, and certified by the Canadian Grain Commission.

Elongase assays of *FAE1* transgenics

Developing seeds were harvested 30 to 35 Days After Pollination (DAP) frozen immediately in liquid nitrogen and stored at -70°C until homogenized. Seeds (approx. 20) were ground in a cold mortar at 0°C in 2 mL grinding buffer (100mM HEPES pH 7.4, 400mM Sorbitol, 2.5mM EGTA, 2.5mM EDTA, 5mM $MgCl_2$, 1mM DTT, PVPP 150 $mg.mL^{-1}$).

The slurried homogenate was filtered through 1 layer of Miracloth and used to perform elongation assays as described by Taylor et al., (1992). In the standard reaction mixture, 0.2 to 0.5 mg of protein was incubated in shaking water-bath (100 rpm) at 30°C for 45 min at pH 7.2 with 90 mM Hepes-NaOH, 1mM ATP, 1mM CoA-SH, 0.5 mM NADH, 0.5 mM NADPH, 2mM $MgCl_2$, 1mM malonyl-CoA + 18 µM [1-^{14}C] oleoyl-CoA ($3.7x10^2$ $Bq{\cdot}nmol^{-1}$) in final volume of 500 µL. In each set of reactions, the amount of homogenate protein added was normalized. Reactions were stopped by adding 3 mL of 100 $g{\cdot}L^{-1}$ KOH in methanol and the mixtures were heated at 80°C for 1 h to saponify the acyl lipids and acyl-CoAs. The tubes were cooled on ice and two-2 mL hexane washes were performed to remove non-saponifiable material. These hexane washes were discarded, and 1 mL water was added to the reaction mixtures. The mixture was then acidified by adding 650 µL concentrated 12 M HCl, extracted twice with 2 mL hexane, the hexane extracts combined and dried under N_2. Samples were transmethylated with 3M methanolic-HCl at 80°C for 1h. 2 mL of 9 $g{\cdot}L^{-1}$ NaCl was added, samples were extracted with 2x with 1 mL hexane, dried under N_2, taken up in 110 µL of acetonitrile and quantified by radio-HPLC as described previously (Taylor et al., 1992b).

Field trials and analysis of progeny

All field trials were conducted by the Saskatchewan Wheat Pool at Rosthern, Saskatchewan (Saskatoon farmzone) in the two successive years. The first field trial growing season (26 May-21 Sept) exhibited 1519 growing degree days, 2309 crop heat units and 172.4 mm of precipitation accumulation. The second field trial growing season (26 May-21 Sept) exhibited 757 growing degree-days, 1278 crop heat units and 167.5 mm of precipitation accumulation (http://www.farmzone.com/report/climate.asp).

In the first field trial, nineteen *SLC1-1* T_3 transgenic lines were field tested in a nursery trial. Transgenics or control lines were planted in a random block design in 3 m rows, with *ca* 100 seeds per row with 60 cm between rows. Data were collected from 2 to 6 rows of each transgenic line and 18 rows of non-transformed Hero control lines.

In the second field trial, 37 *B. napus* cv. Hero *FAE1* transgenic T_2 lines were field-tested in a nursery trial. Transgenics or control lines were planted in a random block design in 3 m rows, with *ca* 100 seeds per row with 60 cm between rows. There were two rows of each line.

In the second field trial, seventeen T_4 *SLC1-1* transgenic *B. napus* cv. Hero lines were selected for yield and quality assessment in the field. The *SLC1-1* yield field trials were of a

random block design. Each plot was *ca* 6 m^2 (5 rows wide at 17.8 cm spacing, and 6 m long in size). The T_4 field-grown lines (leaf material) were sampled and analyzed by PCR to confirm the presence of the 0.95 kb *SLC1-1* insert, using the primers OM087 (5′-AGAGAGAGGGATCCATGAGTGTGATAGGTAGG-3′) (SEQ ID NO:20) and OM088 (5′-GAGGAAGAAGGATCCGGGTCTATATACTACTCT-3′) (SEQ ID NO:21) which were designed according to the 5′ and 3′ end sequences, respectively, of the *SLC1-1* gene as described by Zou et al, (1997).

Analyses were conducted on the progeny (T_5 seed) from triplicate plots. The oil content data collected for each line in this trial were analyzed using the Anova-Fisher's LSD method ($P \leq 0.05$) and Tukey's pairwise comparison method in the Minitab Statistical Software Suite Release 12 (Minitab, Inc. State College PA 16801-3008).

The heterologous expression of the *A. thaliana* seed specific condensing enzyme FAE1 in our target *B. napus* HEA cultivar (cv.) Hero resulted in increased levels of eicosenoic, erucic and total VLCFA in our transgenic lines (Fig.12).

Example 18:

Measure of elongase complex activity in mid-developing seed of *B. napus* cv Hero following heterologous expression of the *Arabidopsis thaliana FAE*.

The *in vitro* assays of elongase activity with homogenates from developing seeds at 30 and 35 DAP (Days After Pollination) from Hero/*FAE1* transgenic lines and Hero wild-type controls showed 22 to 100% increase in total elongase activity in transgenic lines when compared to the wild-type controls (Table IV).

Example 19:

Radio-HPLC measurement of elongase complex activity in mid-developing seed of *B. napus* cv Hero following heterologous expression of the *Arabidopsis thaliana FAE*; Shows *Arabidopsis thaliana FAE* has preference for elongating 18:1 to 20:1.

The reverse-phase HPLC (High Pressure Liquid Chromatography) analyses of transgenic lines and wild-type control lines showed that the amounts of both elongation products eicosenoic acid (20:1) and erucic acid (22:1) were higher in transgenic lines with the amounts of 20:1 elongation product being substantially higher in transgenic lines than in the wild-type controls which confirms the functional expression of *A. thaliana FAE1* gene in transgenic cv. Hero lines and shows that *Arabidopsis* condensing enzyme prefers 18:1 over 20:1, as a substrate.

Example 20:

Transgenic field trials of *B. napus* cv Hero T_3 generation following heterologous expression of the *Arabidopsis thaliana FAE*.

The performance of our Hero/*FAE1* transgenic lines was tested in the field. Thirty seven T_2 transgenic lines were grown in nursery trials. From the GC (Gas Chromatography) fatty acid

methyl ester analyses the erucic acid proportions and oil content of mature T_3 seed from our best transgenic lines showed 8-11% increase in erucic acid proportions and 2-4.8% increase in oil content when compared with the wild type controls (Table V).

Example 21:

Cultivar development of *B. napus* cv Hero: Following heterologous expression of the *Arabidopsis thaliana FAE*, the best performing field trial lines were converted to homozygous doubled haploid lines.

Homozygous lines were produced from selected transgenic lines using microspore-derived embryo technology. The double haploid progeny were analyzed and breeding lines were identified. Seed increases were performed for transgenic field trials and for germplasm development. Using microspore culture technique followed by colchicine treatment doubled haploid lines (DH) were produced from our best Hero/*FAE1* transgenic lines. These lines were grown in the greenhouse under the same growth conditions as wild-type Hero control lines. Our best DH transgenic lines showed stable increases in erucic acid proportions with 58-59% erucic acid, while control lines had on average 48% erucic acid in the seed oil. Hero/*FAE1* transgenic homozygous DH lines and wild-type control lines can be re-tested in transgenic field trials.

Example 22:

Cultivar development of *B. napus* cv Hero: Transgenic field trials of selected DH lines of *B. napus* cv Hero homozygous for the heterologously expressed *Arabidopsis thaliana FAE*.

Ten pure double-haploid (DH) *B. napus* cv. Hero transgenic lines expressing the *A. thaliana FAE1* gene were developed in collaboration with the Saskatchewan Wheat Pool. These transgenic lines were tested in the field by the team of breeders from the University of Manitoba (leading breeder – Peter McVetty). Double-haploid (DH) transgenic Hero/*FAE1* were subjected to field trials planned and conducted by Peter McVetty at the University of Manitoba. The seed was harvested individually from 5 plants in 3 replicates (15 plants total) for each DH line and GC analyses of seed oil content and oil composition were conducted. The results showed that all DH lines have increased erucic acid content in their seed oil (Table VI) with the 5 best lines having the erucic acid content from 7.5 to 8.2% over that found in the wild-type c.v. Hero field-trial-grown control seed. In addition, our best DH Hero/FAE1 transgenic lines have shown increase of up to 7.0% in erucic acid content when compared to c.v. Millenium field-trial-grown control seed (Fig. 14).

Example 23:

Combining the effects of *FAE* transgenes by performing crosses and breeding experiments:

Alternatively, individual *FAE* transgenic lines containing the nasturtium *FAE* gene, or the *Crambe FAE* gene, or combinations of these two *FAE* genes with the *A. thaliana FAE* (ideally homozygous for the *FAE* transgene(s), can be selected and then be used for production of pure,

dihaploid (DH) lines. These DH lines can then be used for crosses and breeding experiments to produce elite HEAR *Brassica* cultivars.

Table I. *Fatty acid composition of transformed tobacco calli.*

Results represent the average (± SE) of tén measurements using independent calli. Constructs: RD= Control (plasmid only) transgenic calli; SF= 35S: *T. majus FAE* transgenic calli; SMF= 35S: mutated *T. majus FAE* transgenic calli.

Construct	Fatty acid composition (% (wt/wt) of total fatty acids) [% increase]*							
	16:0	18:0	20:0	22:0	24:0	26:0	LCFA	VLCFA
RD	20.38 ± 0.12	7.99 ± 0.26	1.32 ± 0.03	0.59 ± 0.03	0.70 ± 0.03	0.89 ± 0.16	96.28 ± 0.31	3.72 ± 0.31
SF	18.01 ± 0.42	5.23 ± 0.41	1.58 ± 0.54 [19.7]	1.32 ± 0.16 [123.9]	1.93 ± 0.27 [175.7]	1.31 ± 0.20 [147.2]	91.37 ± 0.84 (0.84)	8.63 ± 0.84 [131.9]
SMF	19.48 ± 0.34	7.12 ± 0.19	1.30 ± 0.02	0.57 ± 0.03	0.73 ± 0.04	1.01 ± 0.32	95.59 ± 0.40	4.41 ± 0.40 [18.5]

* relative to value for calli from RD: the tobacco control (plasmid only) calli, set at 100%.

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Table II. *Fatty acid composition of transformed tobacco leaves.*

Results represent the average (± SE) of ten measurements using leaf discs from ten independent transgenic plants. Constructs: RD= Control (plasmid only) transgenic leaves; SF= 35S: *T. majus FAE* transgenic leaves; SMF= 35S: mutated *T. majus FAE* transgenic leaves.

Construct	Fatty acid composition (% (wt/wt) of total fatty acids) [% increase]*								
	16:0	18:0	18:3	20:0	20:1c11	22:0	24:0	LCFA	VLCFA
RD	16.32 ± 0.14	3.94 ± 0.11	53.30 ± 0.72	0.53 ± 0.02	1.18 ± 0.00	0.27 ± 0.01	2.74 ± 0.09	93.77 ± 0.29	6.23 ± 0.29
SF	15.83 ± 0.14	3.35 ± 0.12	47.02 ± 0.66	0.91 ± 0.12 [71.7]	2.34 ± 0.12 [98.3]	0.42 ± 0.02 [55.6]	4.14 ± 0.15 [51.1]	88.64 ± 0.35	11.36 ± 0.35 [82.3]
SMF	15.53 ± 0.17	4.00 ± 0.12	47.25 ± 0.85	0.98 ± 0.16 [84.5]	2.61 ± 0.02 [121.2]	0.30 ± 0.01	3.24 ± 0.08 [18.2]	90.05 ± 0.28	9.95 ± 0.28 [59.7]

*relative to value for leaves from RD: the tobacco control (plasmid only) plants, set at 100%.

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Table III. *Fatty acid composition of transgenic Arabidopsis T_2 seeds.*

Results represent the average ± SE of triplicate measurements using 200 seeds from 25 independent *Arabidopsis* transgenic lines.

Constructs: RD= Control (plasmid only) transgenic seeds; NF=Napin: *T. majus FAE* transgenic seeds.

Construct	Fatty acid composition (% (wt/wt) of total fatty acids) {Range} [% increase]*							
	18:0	20:1c11	22:0	**22:1c13**	24:0	24:1c15	LCFA	VLCFA
RD	3.72 ± 0.07 {3.35-4.03}	**19.87** ± 0.26 {17.97-20.86}	0.30 ± 0.01 {0.27-0.34}	**2.12** ± 0.05 {1.88-2.28}	0.11 ± 0.01 {0.09-0.15}	0.19 ± 0.01 {0.15-0.24}	70.15 ± 0.22 {69.35-71.36}	29.85 ± 0.22 {28.64-30.65}
NF	2.57 ± 0.10 {1.58-3.31}	**12.78** ± 0.42 {8.87-16.85}	1.57 ± 0.12 {0.66-2.78} [423.3]	**9.63** ± 0.59 {4.43-15.57} [354.2]	0.46 ± 0.03 {0.24-0.65} [318.2]	0.46 ± 0.03 {0.29-0.70} [142.1]	68.77 ± 0.47 {65.53-72.06}	31.30 ± 0.47 {27.94-34.47} [4.8]

* relative to value for seeds from RD: the *Arabidopsis* control (plasmid only) plants, set at 100%.

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Table IV. Elongase activity in homogenates from developing seed of *B. napus* cv. Hero non-transformed wild-type (H-WT) lines and Hero/*FAE1* T_2 transgenic lines H-10-2 (Assay set 1); H-20-1 (Assay set 2); T_3 transgenic line H-14-7-5 (Assay set 3). Data are the means $\pm$ S.D. from assays of 2 to 5 samples. Homogenates were incubated at 30°C in a water bath with shaking at 100 rpm for 45 min with 18 μM [1–^{14}C] 18:1-CoA ($3.7x10^2$ Bq $nmol^{-1}$) and 1 mM malonyl-CoA in the presence of 1 mM CoA-SH, 1 mM ATP, 0.5 mM NADH, 0.5 mM NADPH and 2 mM $MgCl_2$. After incubation, reaction mixtures were saponified, transmethylated and analyzed by HPLC equipped with a flow through scintillation counter (radio-HPLC).

				Total
	Line	**20:1 Δ11**	**22: 1 Δ13**	**20: 1 Δ11 + 22: 1 Δ13**
		pmol/min/mg protein		
				[% increase] [a]
Set 1	H-WT	493 ± 35	60 ± 3	553 ± 38
	H-10-2	978 ± 43	121 ± 7	1099 ± 50 [99]
Set 2	H-WT	408 ± 48	139 ± 14	547 ± 62
	H-20-1	462 ± 29	203 ± 1	665 ± 30 [22]
Set 3	H-WT	298 ± 22	21 ± 3	319 ± 25
	H-14-7-5	462 ± 6	12 ± 4	474 ± 10 [49]

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Table V. The proportions of erucic acid, total VLCFAs and oil content in seed of non-transformed, *B. napus* cv. Hero wild-type controls (H-WT) and T_3 seed of five selected Hero/*FAE1* transgenic lines (H-10-2 to H-14-7) from field trials.

Line	% 22:1 (w/w)	% Total VLCFAs (w/w)	Oil Content (% of DW)
	[% increase] [a]		
H-WT	48.1	57.6	44.2
H-10-2	59.1 [**22.9**]	67.4 [**17.0**]	47.2 [**6.8**]
H-10-5	56.5 [**17.5**]	64.7 [**12.3**]	46.2 [**4.5**]
H-10-6	56.7 [**17.9**]	64.8 [**12.5**]	46.5 [**5.2**]
H-10-10	57.1 [**18.7**]	65.2 [**13.1**]	44.7 [**1.1**]
H-14-7	56.2 [**16.8**]	63.9 [**10.9**]	49.0 [**10.9**]

[a] Relative to non-transformed wild type Hero control.

Table VI. Proportions of erucic acid and total very long chain fatty acids (VLCFA in DH *B. napus* c.v. Hero/FAE1 transgenic lines and c.v. Hero and c.v. Millennium wild-type control plants from transgenic field trials. The results represent average ±SD of twelve seed samples from ten plants for each transgenic DH line and wild-type (WT) controls. Millenium is an elite commercially grown cv.

Line	22:1 Proportions (% w/w)	VLCFA Proportions (%w/w)
Hero WT control	47.50 ±2.47	59.64 ±1.93
NP00-2978-3	55.46 ±0.52 **[8.0]***	69.05 ±0.98 **[9.4]**
NP00-3030-3	51.80 ±1.67 **[4.3]**	67.42 ±0.89 **[7.8]**
NP00-3091-2	55.03 ±0.63 **[7.5]**	68.77 ±0.40 **[9.1]**
NP00-3094-5	54.87 ±1.10 **[7.4]**	68.79 ±0.49 **[9.1]**
NP00-3098-2	55.43 ±0.26 **[7.9]**	68.98 ±0.33 **[9.3]**
NP00-3115-4	53.67 ±1.96 **[6.2]**	67.98 ±1.59 **[8.3]**
NP00-3171-1	54.65 ±0.32 **[7.1]**	68.81 ±0.33 **[9.2]**
NP00-3190-3	55.35 ±0.91 **[7.8]**	68.86 ±0.35 **[9.2]**
NP00-3193-6	54.36 ±1.59 **[6.9]**	68.65 ±0.51 **[9.0]**
NP00-4498-5	55.69 ±0.63 **[8.2]**	68.56 ±0.30 **[8.9]**
Milennium WT control	48.73 ±2.55	60.57 ±1.55

* **[% increase]** relative to non-transformed wild type Hero control

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DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

Claims

1. An expression vector for transforming a cell, said expression vector comprising a gene coding for a plant fatty acid elongase in reading frame alignment with a promoter capable of increasing expression of said gene, when said transformed cell is in a seed, sufficient to increase the proportion of very long chain monounsaturated fatty acid when compared with a control cell.

2. The expression vector of claim 1 wherein said gene coding for a plant fatty acid elongase is a *Nasturtium* fatty acid elongase gene.

3. The expression vector of claim 1 wherein said gene coding for a plant fatty acid elongase is a *Crambe* fatty acid elongase gene.

4. The expression vector of claim 1 wherein said gene coding for a plant fatty acid elongase is an *Arabidopsis* fatty acid elongase gene.

5. A cell comprising a heterologous gene coding for a heterologous plant fatty acid elongase or allelic variant thereof, said cell being capable of producing an increase in proportion of a very long chain monounsaturated fatty acid when compared a control cell lacking said heterologous gene.

6. The cell of claim 5 wherein said cell is a fungal cell.

7. The cell of claim 6 wherein said fungal cell is a yeast cell.

8. The cell of claim 5 wherein said cell is a plant cell.

9. The cell of claim 5 wherein said cell is a plant seed cell.

10. The plant cell of claim 8 additionally comprising a further heterologous gene coding for an additional heterologous plant fatty acid elongase or allelic variant thereof or a heterologous plant desaturase gene or allelic variant thereof.

11. The plant cell of claim 10 capable of producing oil with an increased content of erucic acid or other very long chain fatty acid (C_{20} or greater).

12. A plant cell according to claim 8 wherein said heterologous gene codes for a 3-ketoacyl-CoA synthase.

13. A plant cell according to claim 8 wherein said very long chain monounsaturated fatty acid comprises erucic acid.

14. A seed comprising a plurality of plant cells according to claim 8.

15. A plant comprising a plurality of plant cells according to claim 8.

16. A plant according to claim 15 wherein said plant is a dicotyledon.

17. A plant according to claim 15 wherein said plant is a member of the *Brassicaceae.*

18. A plant according to claim 15 wherein said plant is a member of the *Limnanthaceae* or *Tropaeolaceae* or *Simmondsia*.

19. A plant according to claim 15 wherein said plant is flax (*Linum usitatissimu* L.).

20. A plant according to claim 17 wherein said plant is of the *Brassica* genus.

21. A method for altering erucic acid content of a plant-derived oil which method comprises cultivating a plant according to claim 15 and then extracting a plant-derived oil therefrom which oil has altered erucic acid content.

22. Use of a heterologous plant fatty acid elongase gene for altering erucic acid content in a plant.

23. Use of a heterologous plant fatty acid elongase gene for altering the very long chain fatty acid content (C_{20} or greater) in a plant.

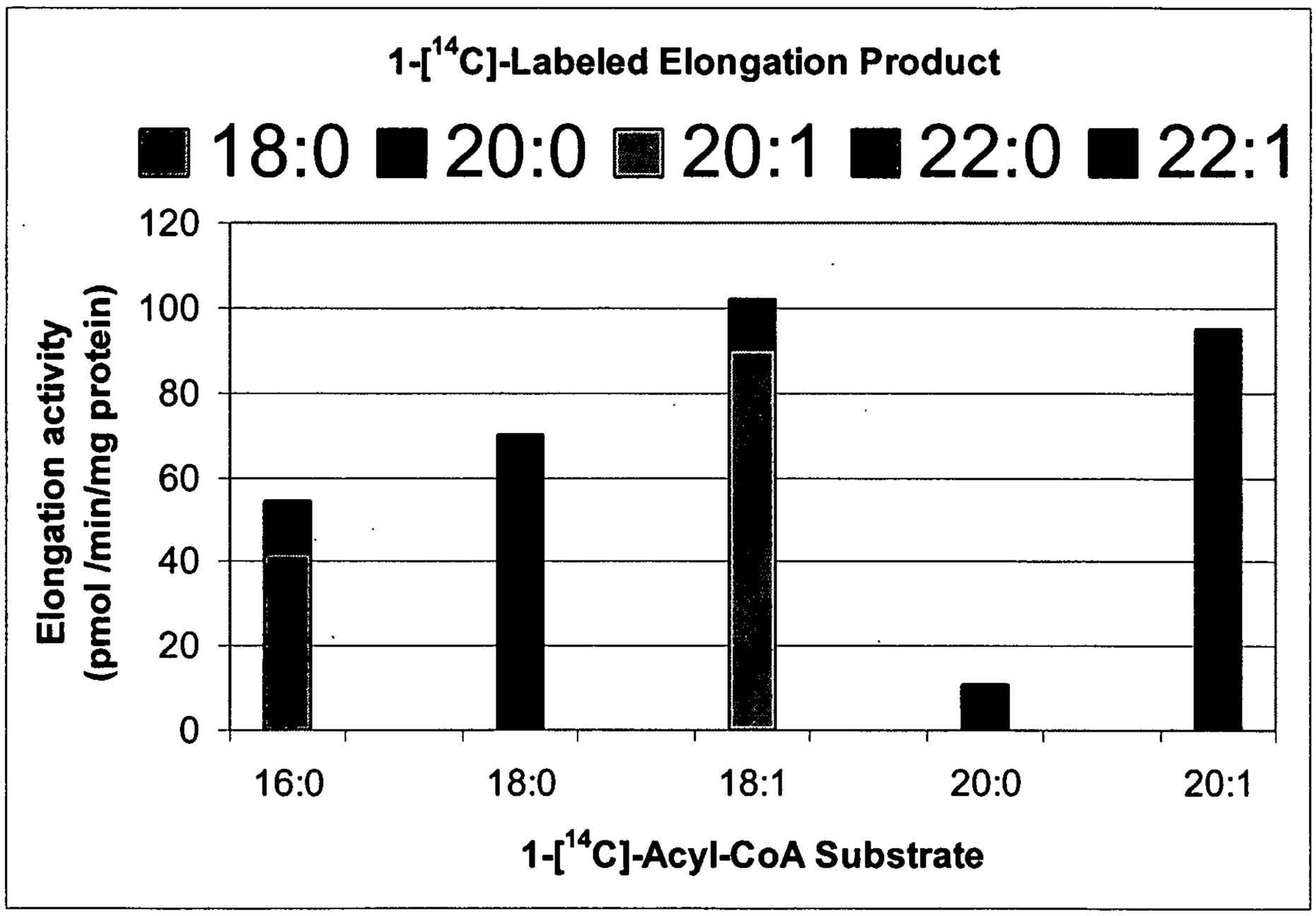

1-[14C]-Labeled Elongation Product
18:0
20:0
20:1
22:0
22:1
Elongation activity (pmol /min/mg protein)
120
100
80
60
40
20
0
16:0
18:0
18:1
20:0
20:1
1-[14C]-Acyl-CoA Substrate

FIG. 1

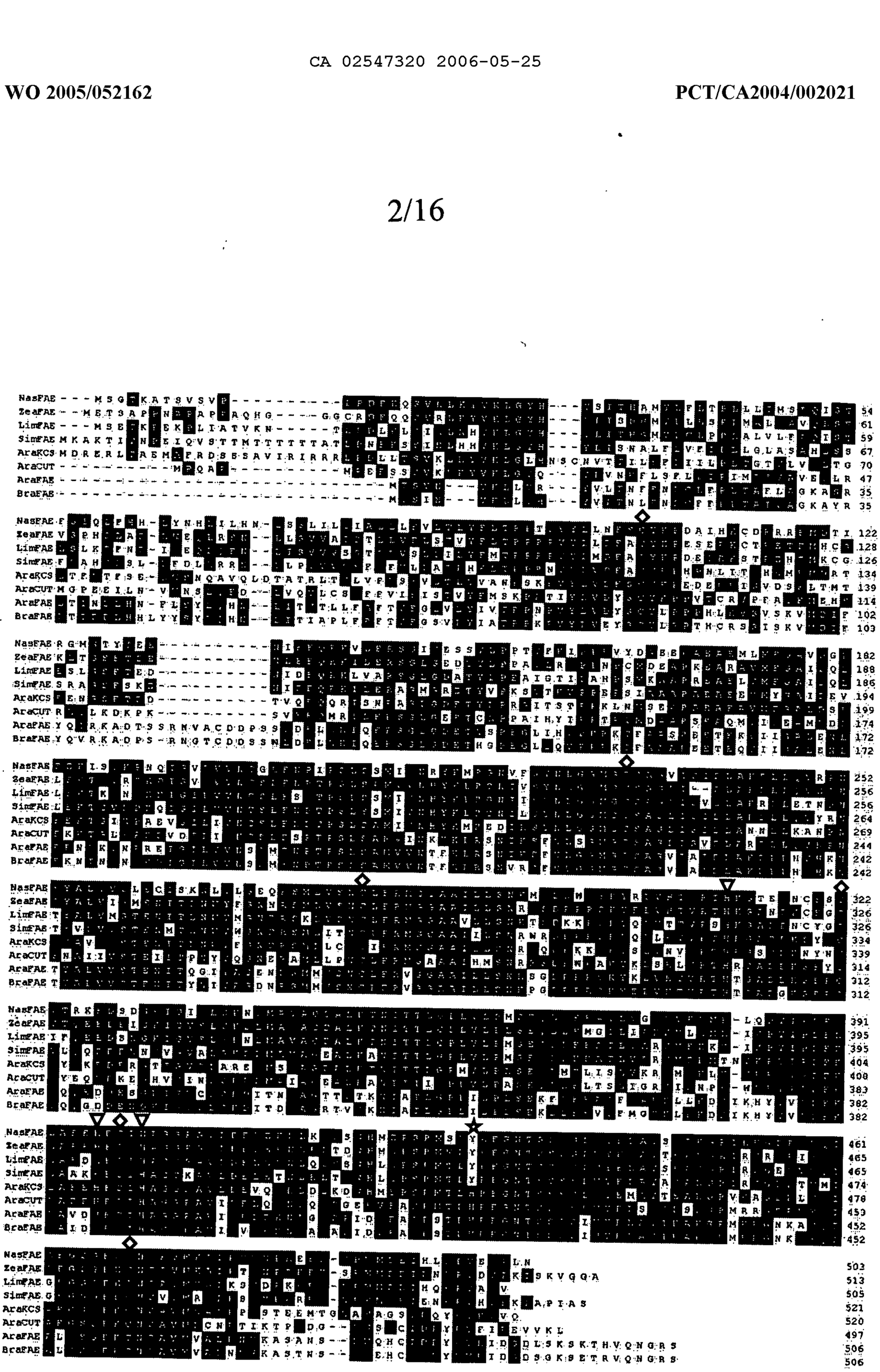


FIG. 2A

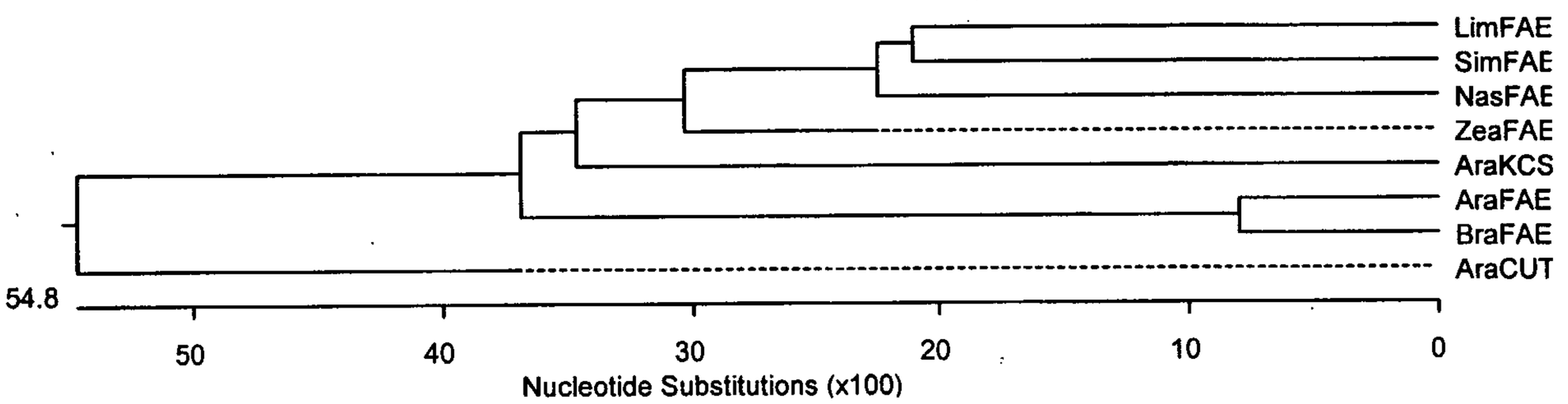

LimFAE
SimFAE
NasFAE
ZeaFAE
AraKCS
AraFAE
BraFAE
AraCUT
54.8
50
40
30
20
10
0
Nucleotide Substitutions (x100)

FIG. 2B

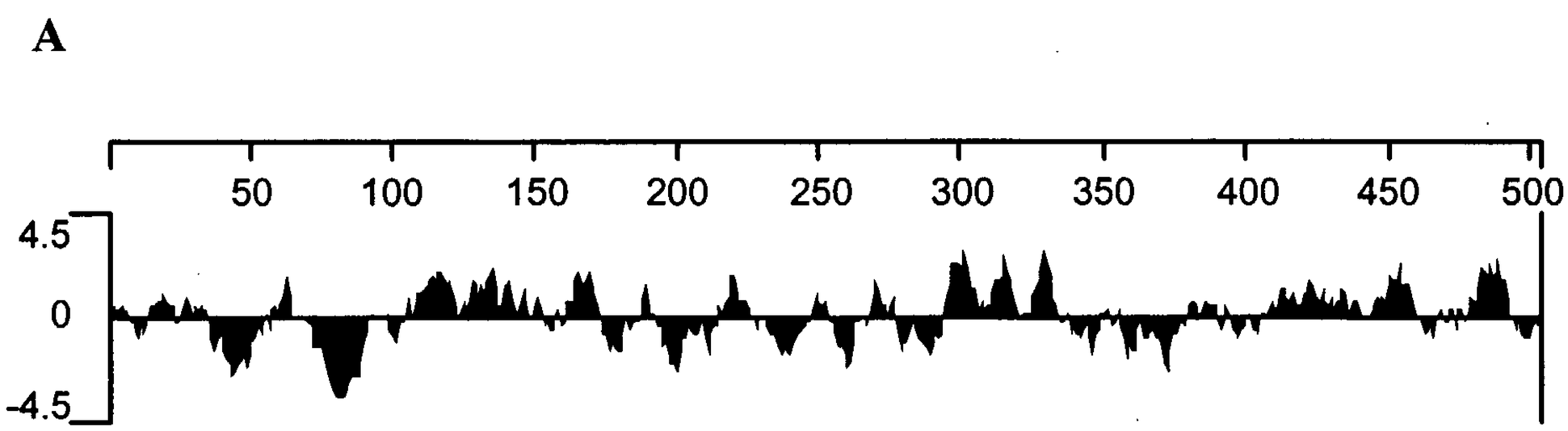
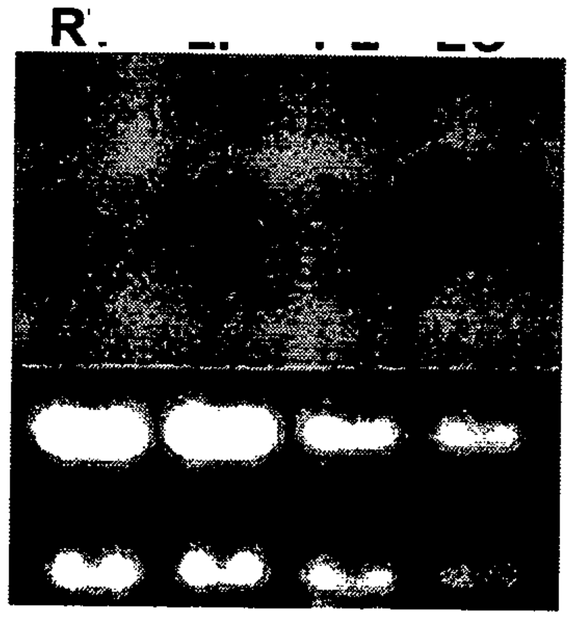

A
50
100
150
200
250
300
350
400
450
500
4.5
0
-4.5
B
Membrane domains
Catalytic domain
35-55
68-88

FIG. 3

A



B



FIG. 4

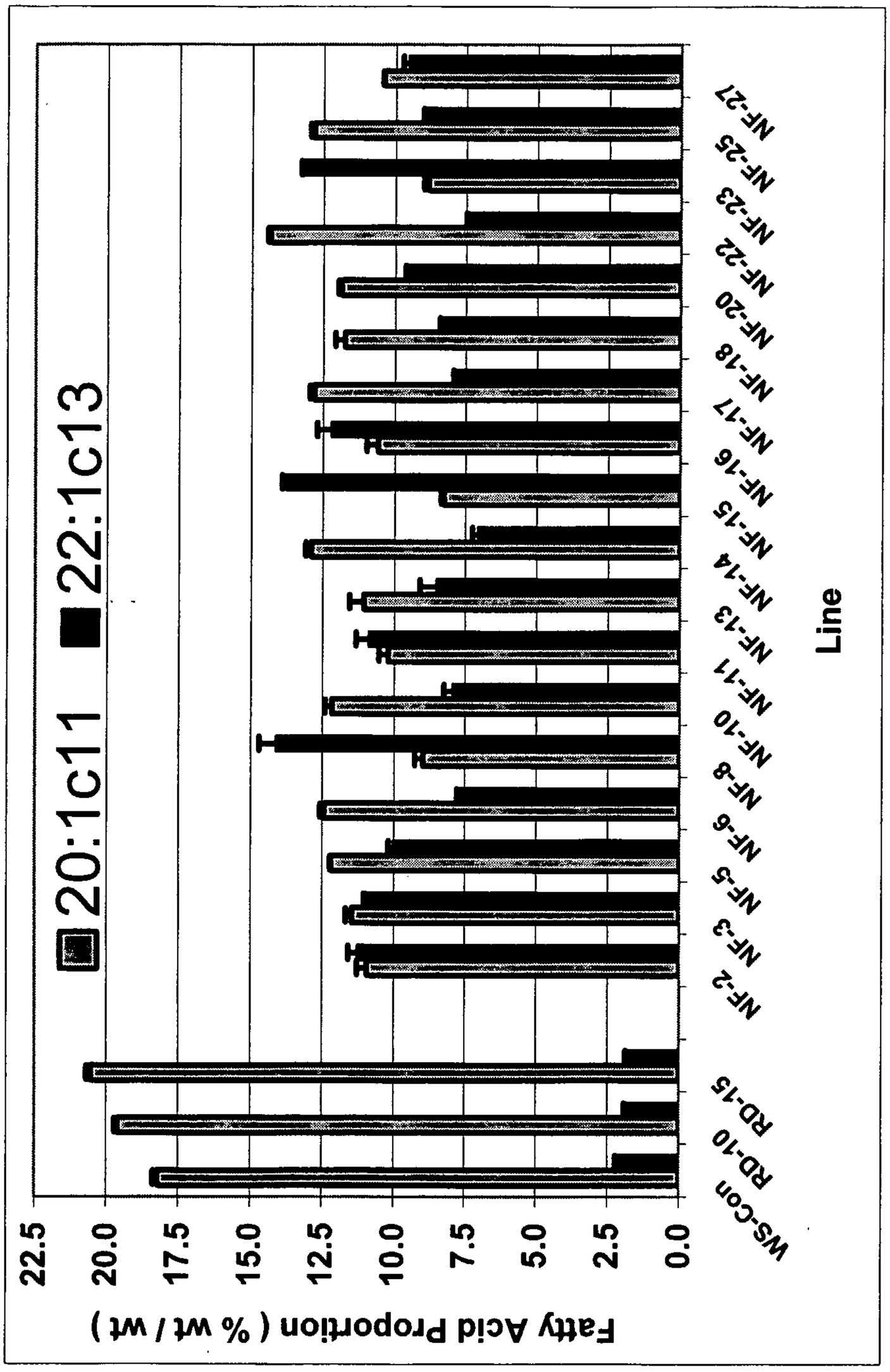

22:1c13
20:1c11
Fatty Acid Proportion (% wt / wt)
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
WS-Con
RD-10
RD-15
NF-2
NF-3
NF-5
NF-6
NF-8
NF-10
NF-11
NF-13
NF-14
NF-15
NF-16
NF-17
NF-18
NF-20
NF-22
NF-23
NF-25
NF-27
Line

FIG. 5A

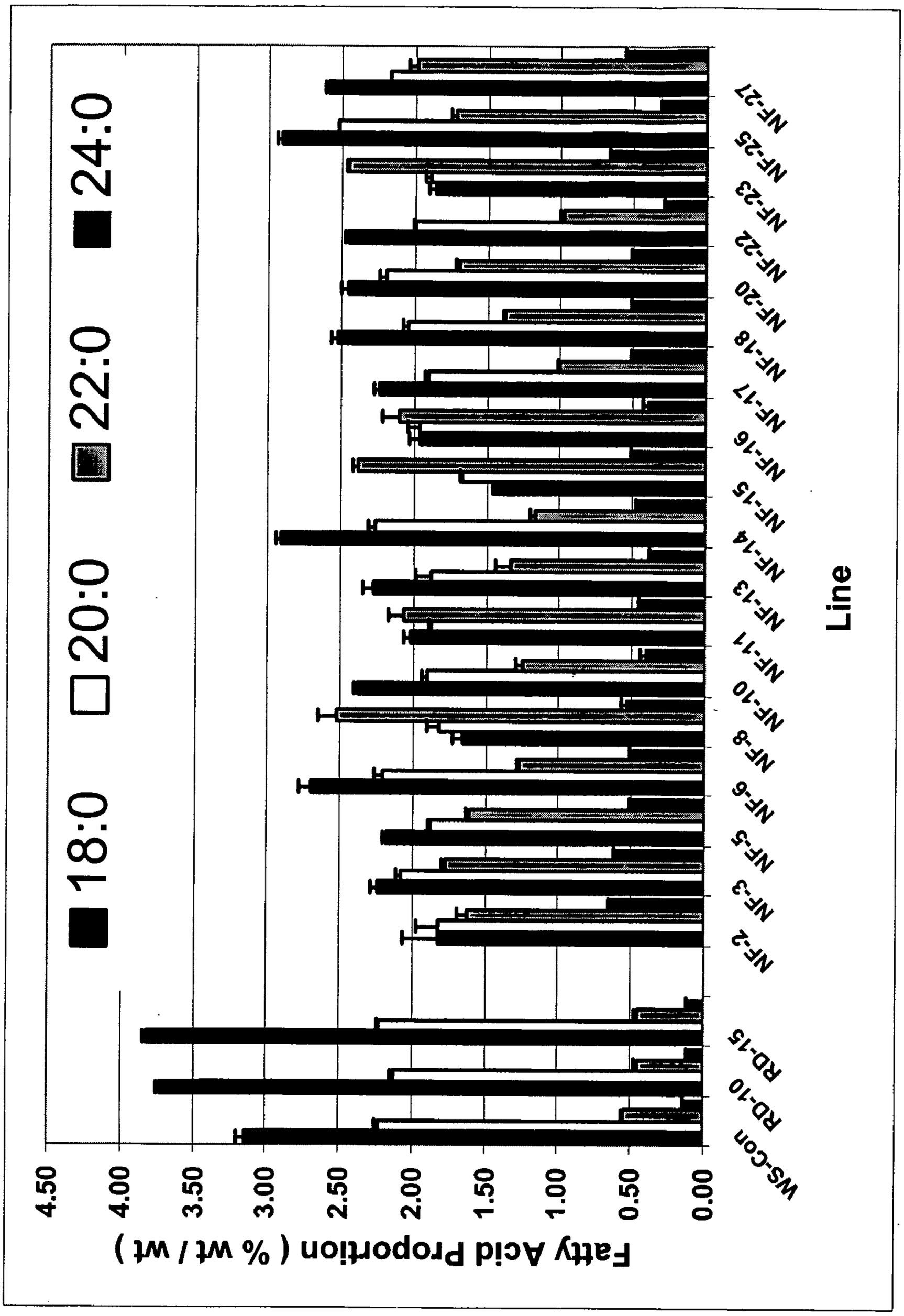

18:0
20:0
22:0
24:0
Fatty Acid Proportion (% wt / wt)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
WS-Con
RD-10
RD-15
NF-2
NF-3
NF-5
NF-6
NF-8
NF-10
NF-11
NF-13
NF-14
NF-15
NF-16
NF-17
NF-18
NF-20
NF-22
NF-23
NF-25
NF-27
Line

FIG. 5B

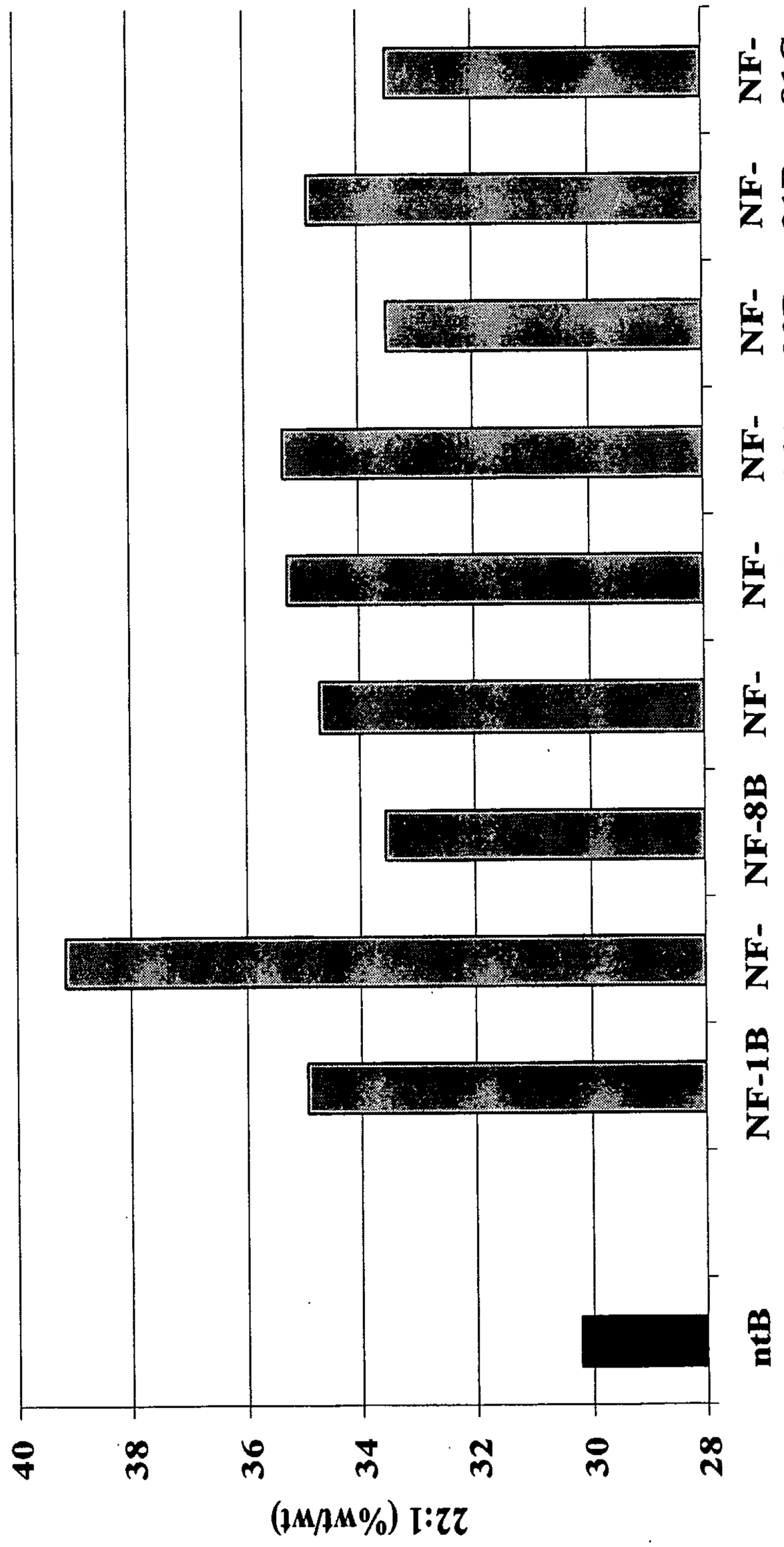

NF-1B
NF-1C
NF-8B
NF-8D
NF-10B
NF-16A
NF-23B
NF-31B
NF-81C
ntB
Line
40
38
36
34
32
30
28
22:1 (%wt/wt)

FIG. 6

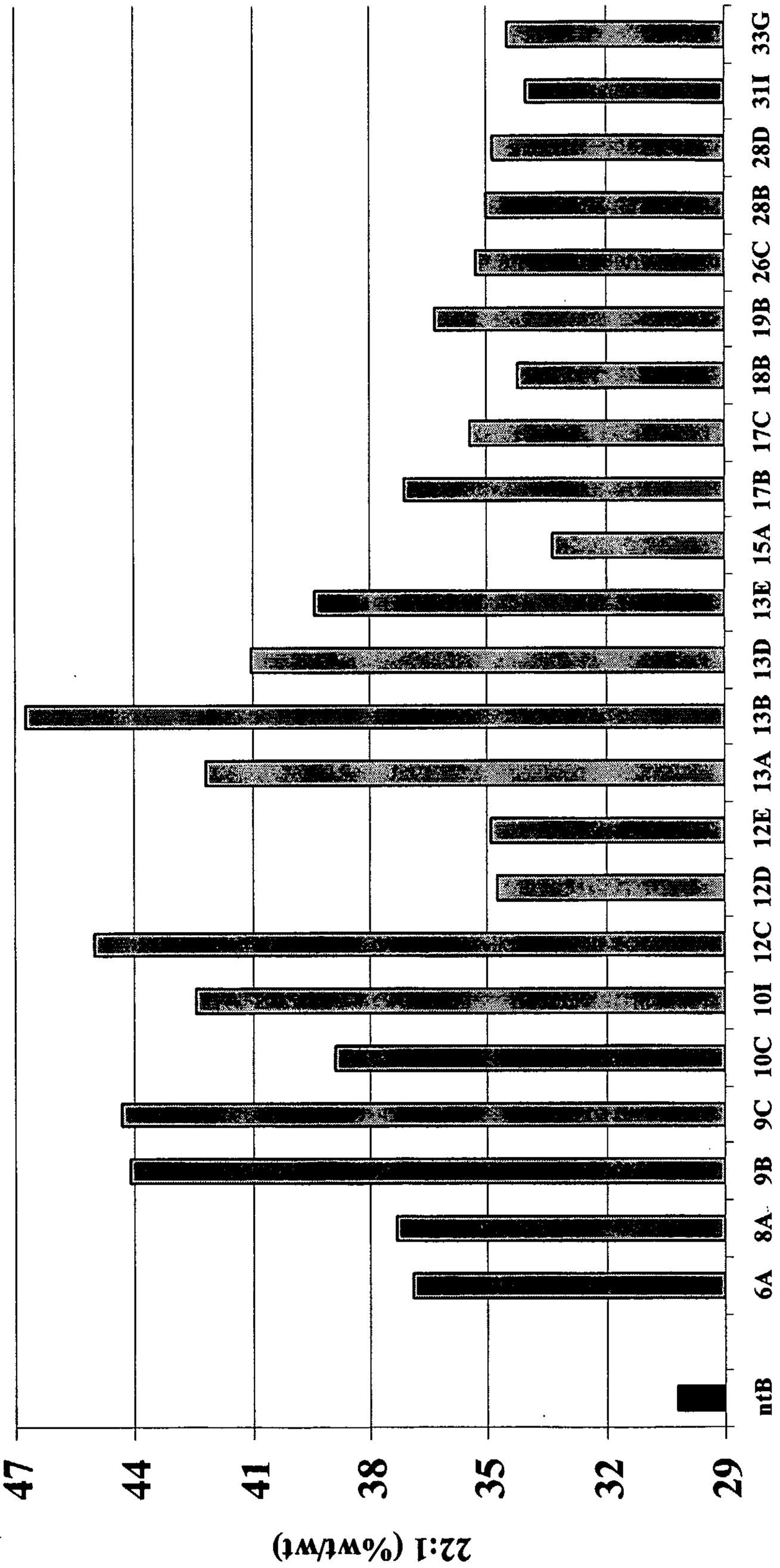

22:1 (%wt/wt)
47
44
41
38
35
32
29
Line
ntB
6A
8A
9B
9C
10C
10I
12C
12D
12E
13A
13B
13D
13E
15A
17B
17C
18B
19B
26C
28B
28D
31I
33G

FIG. 7

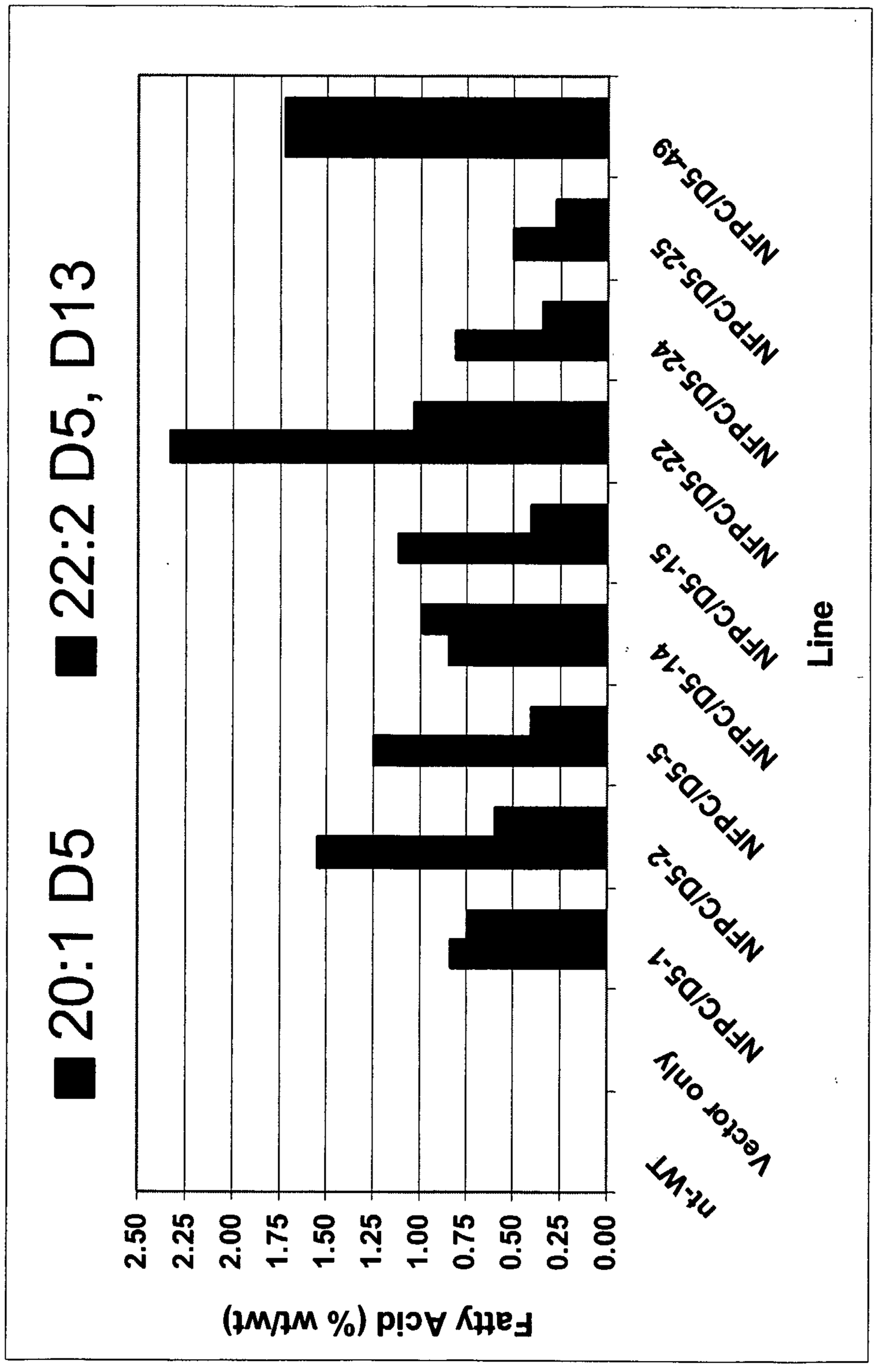

20:1 D5
22:2 D5, D13
Fatty Acid (% wt/wt)
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
nt-WT
Vector only
NFPC/D5-1
NFPC/D5-2
NFPC/D5-5
NFPC/D5-14
NFPC/D5-15
NFPC/D5-22
NFPC/D5-24
NFPC/D5-25
NFPC/D5-49
Line

FIG. 8

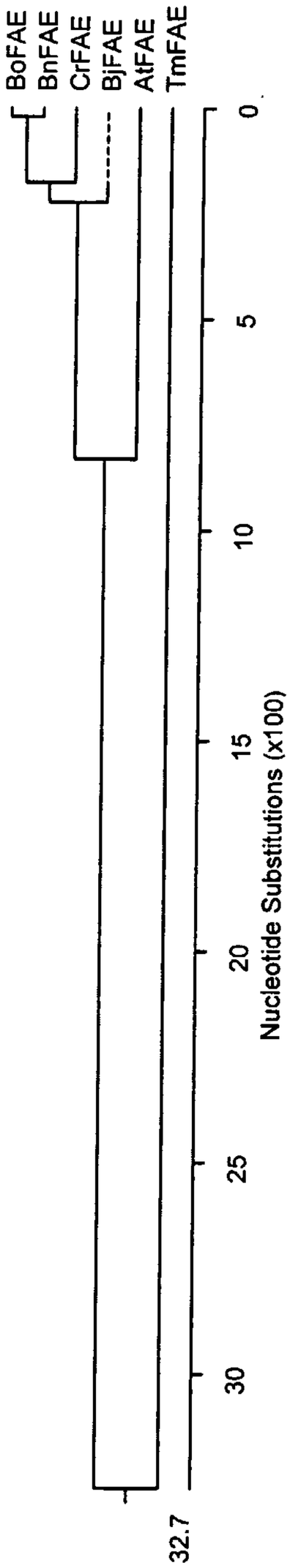

BoFAE
BnFAE
CrFAE
BjFAE
AtFAE
TmFAE
0
5
10
15
20
25
30
32.7
Nucleotide Substitutions (x100)

FIG. 9

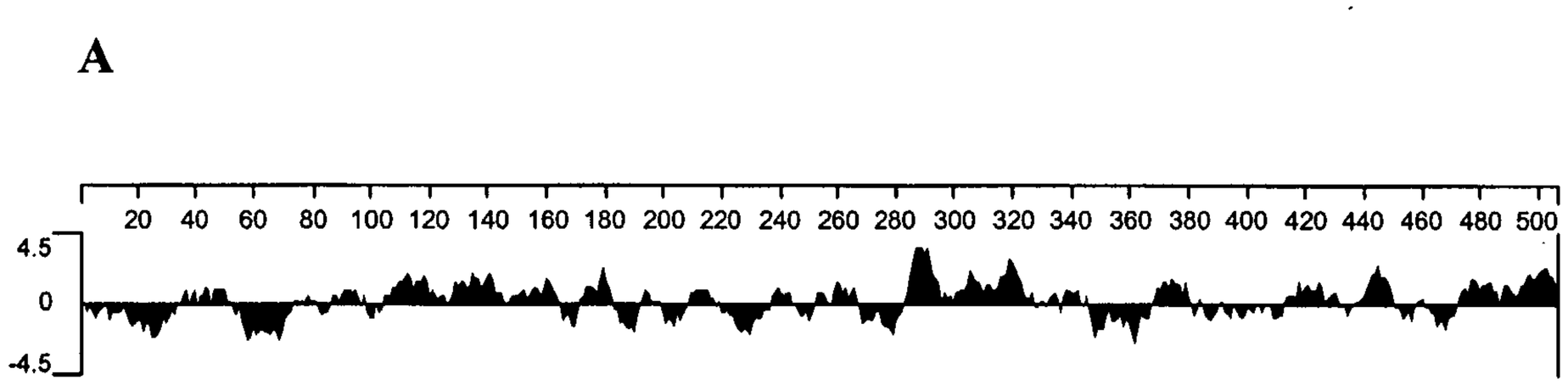

A
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500
4.5
0
-4.5
B
Membrane domains
Catalytic domain
9-31
51-73

FIG. 10

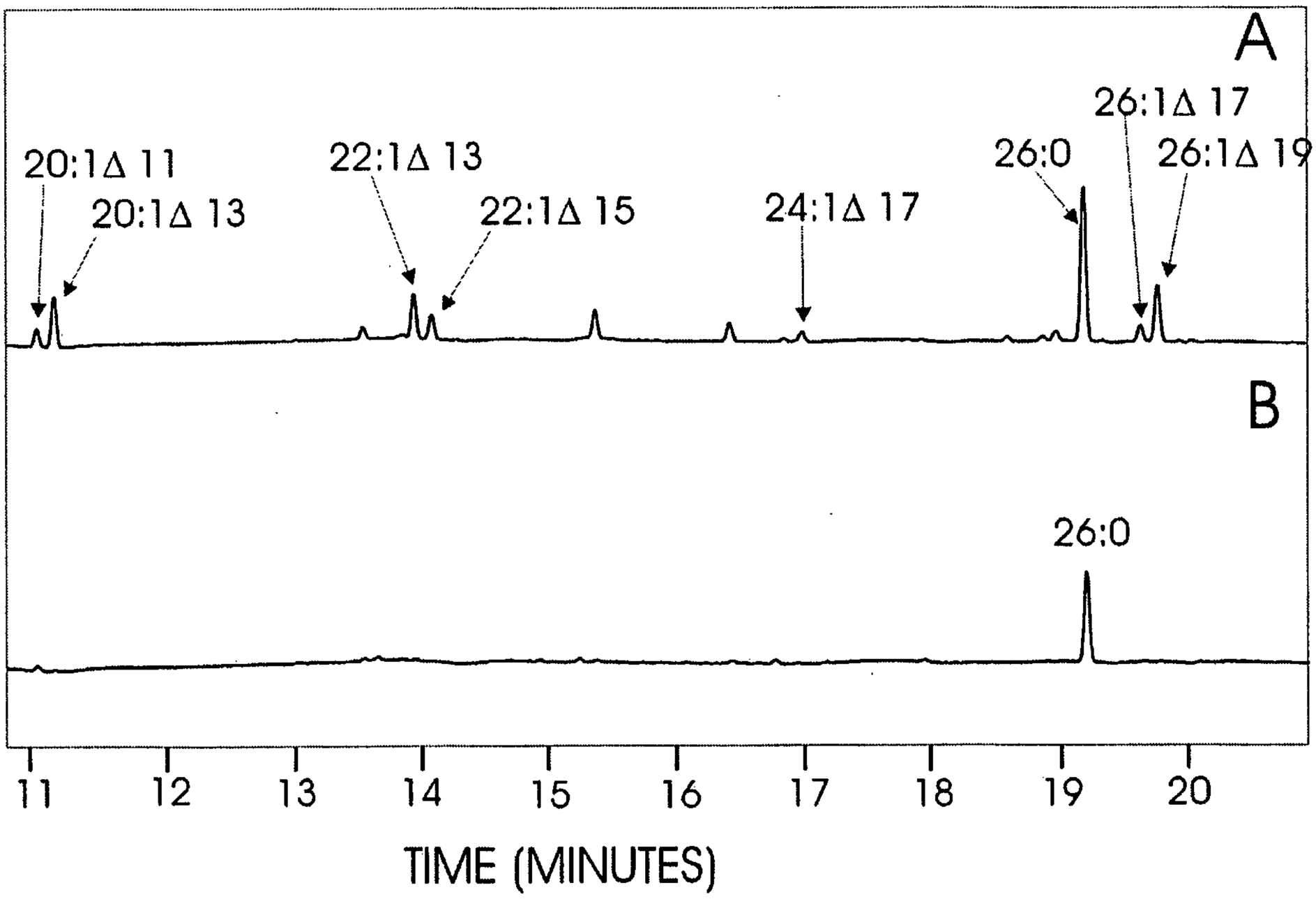

A
20:1Δ 11
20:1Δ 13
22:1Δ 13
22:1Δ 15
24:1Δ 17
26:0
26:1Δ 17
26:1Δ 19
B
26:0
11
12
13
14
15
16
17
18
19
20
TIME (MINUTES)

FIG. 11

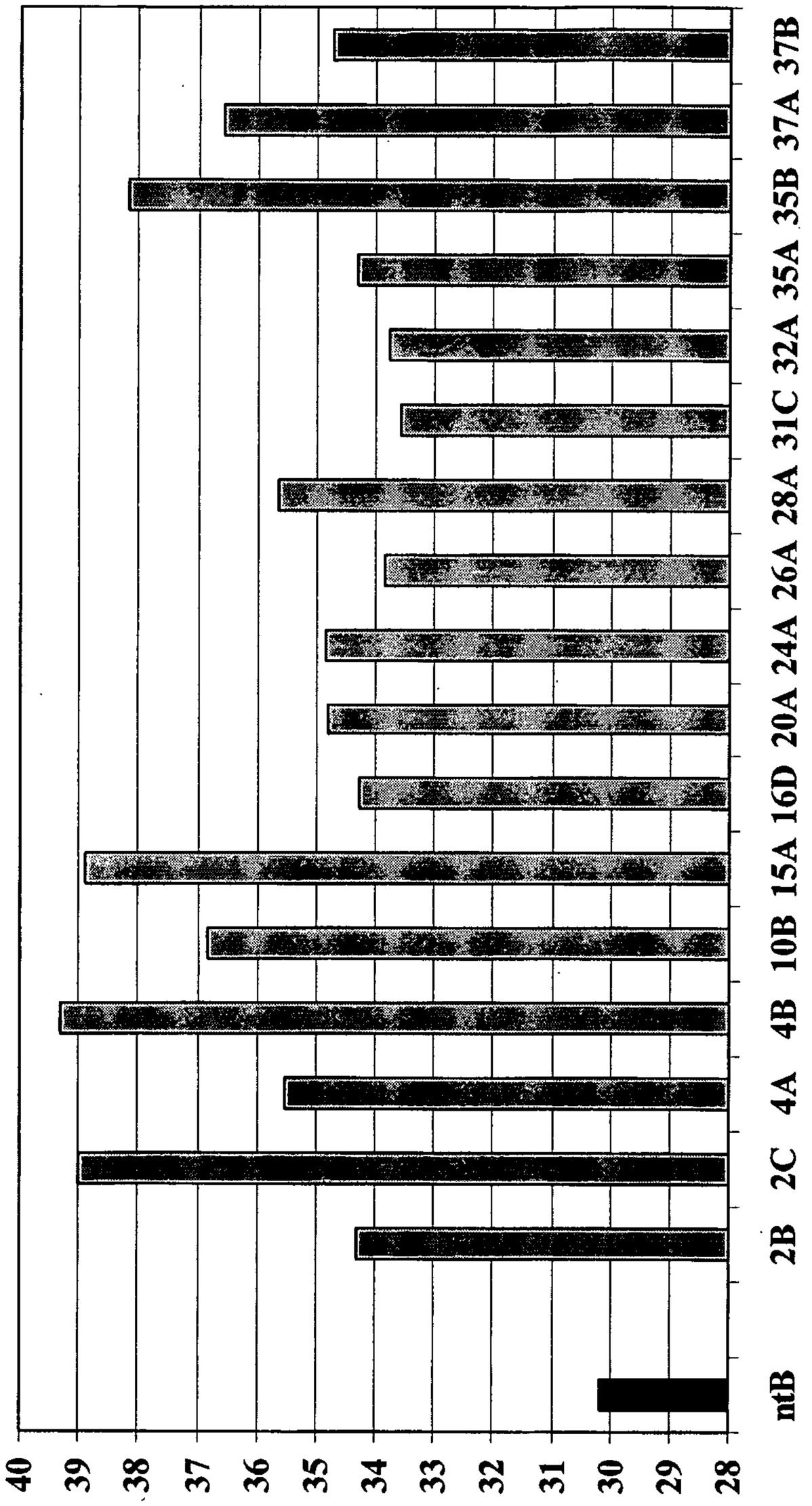

ntB
2B
2C
4A
4B
10B
15A
16D
20A
24A
26A
28A
31C
32A
35A
35B
37A
37B
Line
40
39
38
37
36
35
34
33
32
31
30
29
28
22:1 (%wt/wt)

FIG. 12

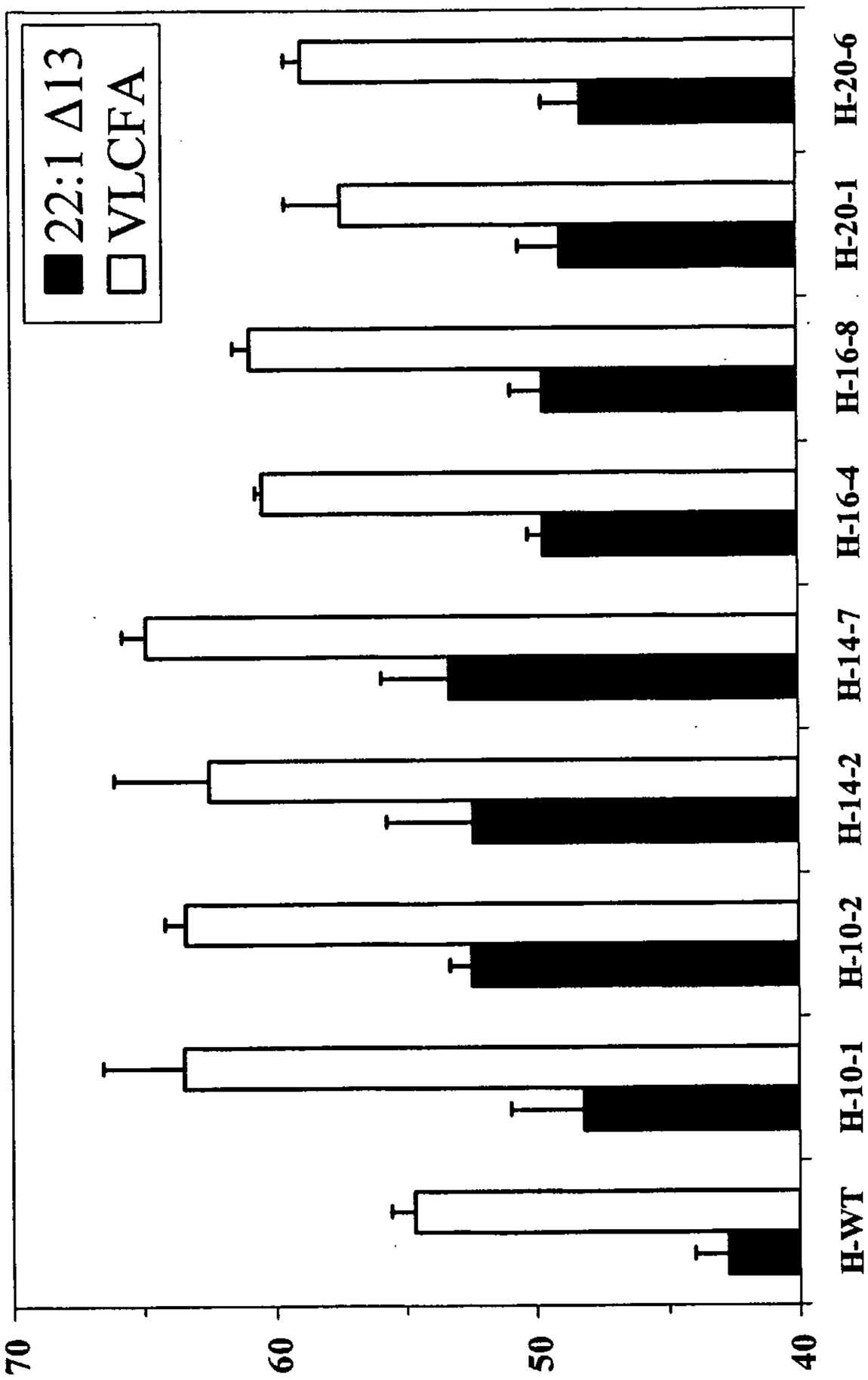

22:1 Δ13
VLCFA
H-WT
H-10-1
H-10-2
H-14-2
H-14-7
H-16-4
H-16-8
H-20-1
H-20-6
Line
70
60
50
40
Fatty Acid (% wt/wt)

FIG. 13

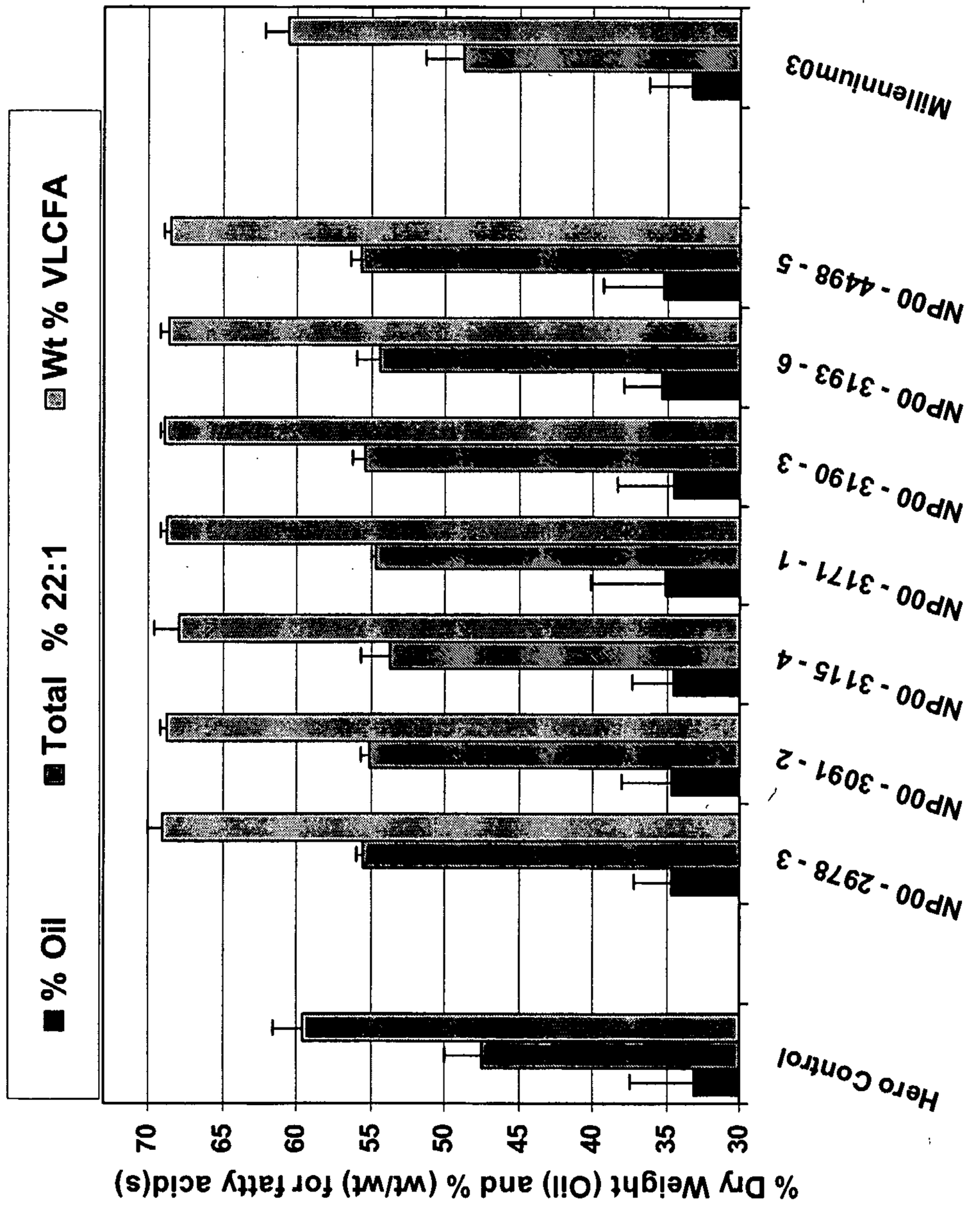


FIG. 14

% Dry Weight (Oil) and % (wt/wt) for fatty acid(s)