New very long chain fatty acid seed oils produced through introduction of strategic genes into Brassica carinata

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TITLE: New very long-chain fatty acid seed oils produced through introduction of strategic genes into *Brassica carinata*

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Note: *The following article is the second in a two-part series. The first part—"Surfactants based on monounsaturated fatty acids for enhanced oil recovery" by Paul Berger—appeared in the September 2010 issue of* inform.

For the emerging global bioeconomy, crop development and enhancement of species diversity are essential. Equally important is maximizing crop value through total crop utilization. We are developing Ethiopian mustard (*Brassica carinata*) as a biorefinery and bioindustrial oils crop platform using traditional and molecular breeding techniques and tools (See Review by Taylor et al., 2010).

Very long chain fatty acids (VLCFA) containing more than 18 carbon atoms are common components of seed oils and plant waxes in a number of plant families including the Cruciferaceae, Limnanthaceae, Simmondsiaceae, and Tropaeolaceae.

Erucic acid (*cis*-docosa-13-enoic acid, 22:1 Δ13) is the major VLCFA in the seed oil from HEAR (high erucic acid rapeseed) *B. napus* cultivars, accounting for 45–55% of the total fatty acids. HEAR cultivars are of high interest since 22:1 is a valuable feedstock with more than 1,000 patented industrial applications. Currently the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an anti-block or slip-promoting agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid, for example, in lubricants, detergents, photographic film processing agents, coatings, cosmetics, and pharmaceuticals.

Studies have confirmed that HEAR oil and its derivatives have a higher energy potential than low erucic acid oil. HEAR oils are more suitable for biodiesel production than low erucic *Brassica* oils, because the iodine value is lower in the former (i.e. lower proportion of poly-unsaturated fatty acids in the oil) and within the European Union specifications. US industry uses 18 million kg of HEAR oil annually, mostly from imports, but supplies are historically limited. Therefore, a large overall market potential exists for expansion and development of new, annually renewable domestic sources of erucic acid oil.

Nervonic acid (*cis*-tetracosa-15-enoic acid; 24:1 Δ15) is another strategic VLCFA. It exists in nature as an elongation product of oleic acid (18:1 Δ9), its immediate precursor being erucic acid. Nervonic acid has been identified in the triacylglycerols in the seeds of only a few plants: *Lunaria* spp. (money plant), borage, hemp, *Acer truncatum* (purpleblow maple), *Tropaeolum speciosum* (flame flower), and *Cardamine graeca* (bittercress). Nervonic acid is particularly abundant in the white matter of animal brains and in peripheral nervous tissue where nervonyl sphingolipids are enriched in the myelin sheath of nerve fibers.

Interest in dietary therapy with nervonic acid-containing fats and oils developed when a hypothesis was put forward that dietary nervonic acid could support the normal synthesis and functionality of myelin in brain and nerve tissues. Dietary supplementation with nervonic acid might be beneficial, for neurological development/function, in: (i) individuals with genetic disorders of lipid metabolism specifically associated with peroxisomes (adrenoleukodystrophy, Zellweger's syndrome, others); (ii) individuals with multiple sclerosis and other nervous disorders such as Parkinson's disease; and (iii) infants, particularly prematurely born infants, receiving formula as a source of nutrition. Realization of these potential nutritional applications is limited by the lack of available sources of a nervonic acid-rich oil that has minimal amounts of erucic acid. Provided the erucic acid content is very low, dietary intake of oil with high proportions of nervonic acid is predicted to be nontoxic to humans and animals. Nervonic acid is therefore a strong candidate for further evaluation as a bioactive lipid supplement, similar to arachidonic acid, docosahexaenoic acid, and conjugated linoleic acids, for the promotion of human and animal health (See Review by Taylor et al., 2009). Surprisingly, nervonic acid also has applications as an industrial feedstock, in a manner similar to erucic acid.

A strategic goal of our research is to modify seed oil composition in members of the Brassicaceae to increase the proportion of VLCFA. Specific genetic modifications have been made to produce *B. carinata* prototype lines delivering oils highly enriched in VLCFAs ; in addition we have used transgene technology to enhance *B. carinata* seed oil content, which is 5–7% lower than *B. napus* (canola and HEAR cultivars). While we have HEAR in western Canada and European cultivars that are grown in winter, we are advocating that *B. carinata* be developed as an alternative crop platform for industrial oil production and high VLCFA oils, in particular on the Canadian prairies (Fig. 1). *Brassica carinata* is genetically transformed at a very high efficiency; the crop is highly disease resistant (e.g., to the fungus blackleg), is drought-tolerant, and is amenable to growth in hotter, drier regions.

Total crop utilization can be illustrated with two examples: High allyl glucosinolate meal from *B. carinata* can be used directly as a biopesticide; alternatively, the meal can be processed with a solvent wash to remove the glucosinolates so that the remaining low-fiber meal can be used as fish feed. We are currently working closely with *B. carinata* breeders at Agriculture and Agri-Food Canada, Saskatoon, to make sure that we can cross many of our oil-quality enhanced traits into their elite breeding lines.

FIG. 1. B. carinata *in experimental field plots.*

Some of the many industrial and health-dietary supplement-related uses of VLCFA oils are shown in Table 1 and Tables 2 and 3, respectively**.** Specifically, the high-VLCFA feedstocks are essential components of new products developed by Paul Berger and associates at Oil Chem Technologies, Inc. (Sugar Land, Texas, USA), which include viscoelastic and high molecular-weight anionic surfactants. Some of these products show great potential in enhanced oil recovery (EOR) strategies as described recently in *inform* (*21*:542–543, 592, 2010).

TABLE 1. Industrial applications of oils highly enriched in VLCFAs[a]
Viscoelastic surfactants and high molecular weight anionic surfactants
Enhanced oil recovery surfactants
Paving bed polymers
Erucamide—slip-promoting, anti-blocking agent in manufacture of plastic films
Nylon 13,13 or Nylon 15,15
Polyurethanes, plastics, and foams
Coatings and adhesives
Modified epoxide gels and resins
Composite materials
Cosmetic formulations
Silver behenate emulsions to be used in film processing
High-temperature lubricants

[a]VLCFAs, very long chain fatty acids

TABLE 2. Potential pharmacological applications of high nervonic acid oils
Demyelinating diseases such as multiple sclerosis (MS) and adrenoleukodystrophy (ALD): There is a marked reduction in nervonate sphingolipids in post-mortem MS and ALD patients.
Parkinsonian tremors: Defects in microsomal biosynthesis of VLCFA including nervonate in "jumpy" and "quaking" mice model systems are accompanied by impaired myelination.
Schizophrenia: Nervonic acid is very low in post-mortem brain matter in these patients.
HIV: Nervonic acid has been reported to inhibit HIV-1 reverse transcriptase activity in a dose-dependent manner.

TABLE 3. Potential applications in dietary supplementation using high nervonic acid oils
Infants or young children during the key "myelinating stage" of neurodevelopment (*ca*. up to age 5), e.g. baby food and infant formula supplementation
Supplements for women who intend to become pregnant, are pregnant, or are lactating (particularly important with pre-term babies)
High-level training/exercising adults whose nervonic acid levels are generally taken to be normal; provides neuroprotective effect
Supplement in cattle feed e.g. to enrich cows' milk in 24:1 for provision of milk products to infants and adults; 24:1 partitions with the protein fraction in dairy processing (not the typical fat fraction) and thus it is available in non-fat dairy and whey products

Most transformation systems for introducing new genes into *B. carinata* rely on *Agrobacterium*-mediated infection of cotyledon (first leaf) petiole tissue with a genetic cassette containing the gene or genes of interest, promoter and terminator elements, and a selectable marker—usually a gene conferring resistance to a specific antibiotic or herbicide. The latter makes it possible to "screen" the putative transgenic petiole pieces for positive incorporation and integration of the cassette into the target plant tissue. Then the positive transgenic tissues are regenerated into whole transgenic plants by growth on root- then shoot-promoting medium and transferred to soil. These were the methods we used to introduce the transgenes into *B. carinata* as described below.

TARGET TRANSGENES AND BIOPRODUCTS

To enhance the 22:1 or 24:1 acid content of *B. carinata* oil, we selected two nontraditional sources of genes encoding ketoacyl-CoA synthases(KCSs), the KCS being the first of four enzymes in an elongase complex that ultimately converts 18:1 Δ9 (oleoyl)-CoA to erucoyl-CoA and then nervonoyl-CoA by successive cycling of the complex, with malonyl-CoA providing the two carbons needed for each extension cycle. These gene sources were *Crambe abyssinica* and *Cardamine graeca* (Fig. 2).

FIG. 2. Left: Crambe abyssinica *(Abyssinian mustard), source of a ketoacyl-CoA synthase (KCS) gene expressed in* B. carinata *to enhance seed oil erucic acid content.* ***Right:*** Cardamine graeca *(bittercress), source of a* KCS *gene expressed in* B. carinata *to create a high nervonic acid oil.*

When introduced into *B. carinata*, *Crambe KCS* and *Cardamine KCS* genes resulted in seed oils highly enriched in erucic acid and nervonic acid (Figs. 3 and 4, respectively).

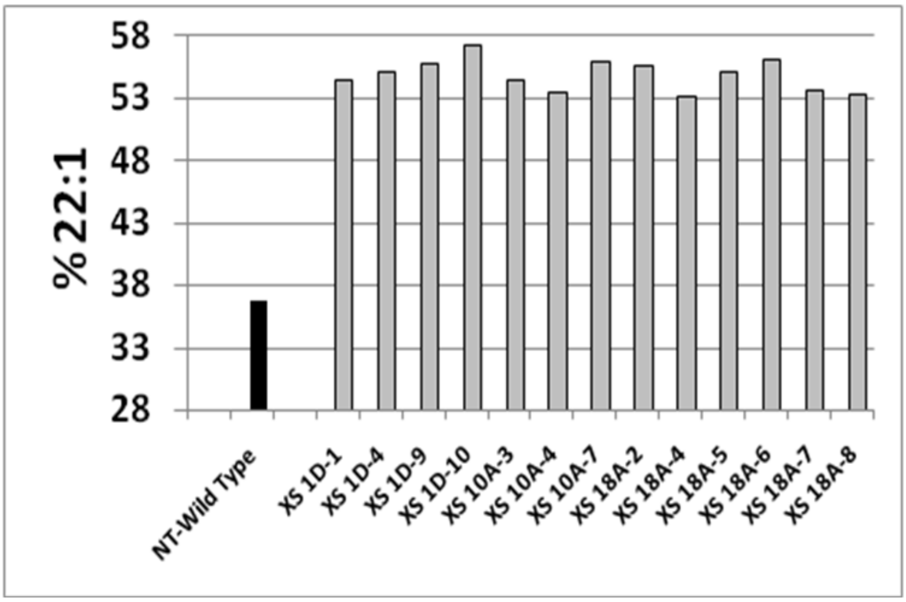

FIG. 3. *Erucic acid (22:1) proportions (% wt/wt) in mature seeds of non-transformed* B. carinata *wild type control (NT-Wild Type, black bar) and T_2 seeds of* B. carinata *transgenic lines carrying the* Crambe KCS *(lines XS 1D-1 through XS 18A-8; grey bars*) *under control of the seed-specific napin promoter*.

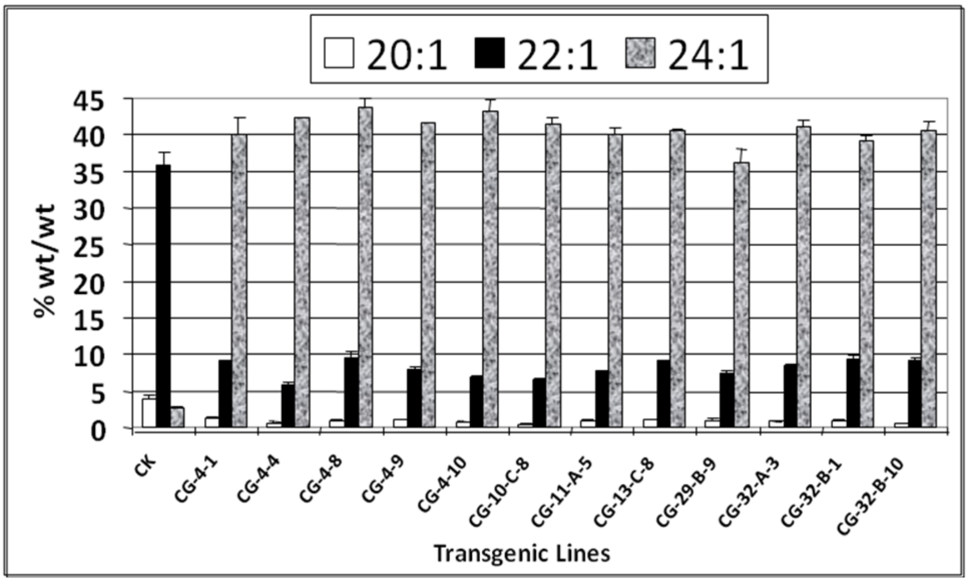

FIG. 4. *Fatty acid composition of T_3 seed oil of transgenic* B. carinata *transformed with the* Cardamine graeca KCS *gene. Proportions of 20:1 (white bars), 22:1 (black bars), and 24:1 (grey marbled bars) in seed oils from empty plasmid-only transgenic control line (CK), and the twelve best* B. carinata *T_3 transgenic lines (lines CG-4-1 through CG-32-B-10) expressing the* C. graeca KCS *gene (CG) under control of the seed-specific napin promoter.*

OUTLOOK FOR *B. CARINATA* AS A PLATFORM CROP FOR DELIVERY OF BIOPRODUCTS

When considering potential platform crops for the delivery of bio-oils and industrial feedstocks, seed yield and oil and protein content are major considerations. Consequently, the plant must be more efficient in resource utilization than the usual cultivars while maximizing yield. *Brassica carinata* delivers high yields among the Brassicaceae (up to *ca* 2,500 kg/ha). A key component of any new crop development is the question of whether it contributes positively to sustainable agriculture. This fledgling crop platform can meet or exceed many of the targets for sustainable agriculture. Specifically:

- Plant-produced VLCFA oils provide renewable, biodegradable, non-fossil fuel feedstocks for the production of polymers, plastics, waxes, pharmaceutical and nutraceutical oils.
- For example, high nervonic acid oil from *B. carinata* can find direct applications equally in polymers, paving substances, and surfactants for oil recovery/reclamation products, as well as potential new products for enhancing infant nutrition and fighting the symptoms of neurodegenerative diseases.
- *Brassica carinata* is well suited for growing in the drier southern regions of western Canada.
- Creation of a new crop platform adds genetic diversity. It creates a new delivery system for bioindustrial and pharmaceutical oils that do not impact/compete with the food sector, specifically canola.
- Growing B. carinata in areas not suitable for canola means that one is adding a new oilseed to crop rotations in those areas.
- *Brassica carinata* requires fewer inputs owing to its natural resistance to drought and blackleg; more robust architecture means crop stands are able to better compete with weeds.
- *Brassica carinata* provides the grower with enhanced yield (kg/ha) compared with other Brassicas (canola) and is therefore an attractive incentive for farmers as it could result in increased returns at the farmgate.
- The unique characteristics of *B. carinata* meal provide new opportunities as feedstocks for plastics and antigen delivery systems; the utility of both oil and meal is essential for complete utilization of the seed products, providing greater sustainability.

In conclusion, as indicated by the two case studies discussed above, *B. carinata* is well suited for genetic engineering, and the generation of transgenics will play a major role in designing this crop for the delivery of VLCFA-enhanced oil feedstocks for bio-products.

Selected Reviews from this author:

Taylor DC, Guo Y, Katavic V, Mietkiewska E, Francis T and Bettger W (2009) New Seed Oils for Improved Human and Animal Health and as Industrial Feedstocks: Genetic Manipulation of the *Brassicaceae* to Produce Oils Enriched in Nervonic Acid**. *In: Modification of Seed Composition to Promote Health and Nutrition*, ed Krishnan HB, ASA-CSSA-SSSA Publishing, Madison, WI, pp 219-233.**

Taylor DC, Falk KC, Palmer CD, Hammerlindl J, Babic V, Mietkiewska E, Jadhav A, Marillia E-F, Francis T, Hoffman T, Giblin EM, Katavic V and Keller WA (2010) *Brassica carinata* – a new molecular farming platform for delivering bio-industrial oil feedstocks: case studies of genetic modifications to improve very long-chain fatty acid and oil content in seeds. ***Biofuels, Bioproducts and Biorefining* 4: In Press; published online Aug 2, 2010** DOI: 10.1002/bbb.231**.**

Dr. David Taylor has been active for 22 years in the field of oilseed biochemistry, biotechnology, and oil modification at the National Research Council of Canada Plant Biotechnology Institute in Saskatoon, Saskatchewan. His research focuses on the creation of value-added oils as industrial feedstocks or for human consumption using biotechnology. He is also actively involved in research to improve the oil content of a number of oilseed crops including canola, Brassica carinata *and flax. He is a strong advocate for adoption of* B. carinata *as a new platform crop for delivery of customized bioindustrial oils. Email him at David.Taylor@nrc-cnrc.gc.ca* This is NRCC publication no 50172.