Brassica carinata-A New Molecular Farming Platform for Delivering Bio-industrial Oil Feedstocks: Case studies of Genetic Modifications to Improve Seed Very Long-Chain Fatty Acid and Oil Content in Seeds

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Review

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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†

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Abstract: Crop development and species diversity are important aspects of the emerging global bioeconomy, as is maximizing crop value through total crop utilization. We advocate development of *Brassica carinata* as a biorefinery and bioindustrial oils platform using traditional and molecular breeding techniques and tools. We review genetic studies and breeding efforts to develop elite *B. carinata* germplasm, work involving development of transformation

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and regeneration protocols, target gene isolation, and transgene expression. Genetic modification strategies using a *B. carinata* breeding line as a delivery platform for very long-chain fatty acid-enhanced/modified oils are presented as case studies. The target oil products are erucic acid (22:1 Δ13), docosadienoic acid (22:2 Δ5, Δ13) and nervonic acid (24:1 Δ15); in addition transgenic efforts to enhance *B. carinata* seed oil content are discussed. The overall advantages and current limitations to utilizing this crop are delineated. Other anticipated biobased products from a *B. carinata* platform may include, but are not limited to, the production of biolubricants, biofuels and biopolymers from the oil, biopesticides, antioxidants, as well as plant gums, and vegetable protein-based bioplastics and novel food and feed products. In summation, this collaborative *B. carinata* breeding/germplasm development/value-added molecular modification effort will not only contribute to the development of renewable feedstocks for the emerging Canadian bioeconomy (biorefinery/bioproducts), but also promises to generate positive economic and environmental benefits. Published in 2010 by John Wiley & Sons, Ltd.

Keywords: *Brassica carinata*; genetics; breeding; genetic modification; industrial and nutraceutical/pharmaceutical oils and applications

Abbreviations: CTPase, CDP-choline:diacylglycerol cholinephosphotransferase (EC 2.7.8.2); DGAT, diacylglycerol acyltransferase (EC 3.2.1.20); Docosadienoic acid (22:2 Δ5, Δ13); FAE, fatty acid 4-enzyme elongase complex; Erucic acid (22:1 Δ13);GPAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); KCS, 3-ketoacyl-CoA synthase (condensing enzyme) (EC 2.3.1.85); LPAT, *lyso*-phosphatidic acid acyltransferase (EC 2.3.1.51); LPCAT, *lyso*-phosphatidylcholine acyltransferase (2.3.1.23); Nervonic acid (24:1 Δ15); PAPase, phosphatidic acid phosphataste (EC 3.1.3.4); PDAT, acyl-CoA-independent phosphatidylcholine (EC 2.3.1.158); SLC1-1, *Saccharomyces cereviseae* LPAT

Introduction

As the global demand for vegetable-based oil and protein products increases, breeders are faced with the task of developing new and improved oilseed germplasm to expand the current acreage. The major canola species, *Brassica napus* (double low erucic and glucosinolate) is adapted to the cool moist northern growing areas of western Canada but has limited use in hotter and drier regions. *Brassica rapa* is well adapted to both areas but is lower yielding and is not currently available in an herbicide tolerant form. Although, the development of canola-quality *B. juncea* will undoubtedly contribute to the expansion of oilseeds in drier regions, *B. carinata* also has the potential to increase production in these areas.

Brassica carinata, commonly called Abyssinian or Ethiopian mustard or known locally as gomenzer, is an amphidiploid (BBCC, 2n = 34) formed through interspecific hybridization between the diploid species *B. nigra* L. (BB, 2n = 16) and *B. oleracea* L. (CC, 2n = 18).[1] Ethiopian mustard is well adapted to the highland areas of Ethiopia where it is grown for its leaves, which are plucked, boiled, and eaten, and for the edible oil in the seed. There is little or no commercial production of this species outside of Ethiopia or neighboring countries. Ethiopian mustard is highly heat and drought tolerant, has good resistance to blackleg disease,[2] resistance to aphids and flea beetles,[3] relatively large seed size[4] and some accessions are resistant to alternaria black spot.[5]

This species is genetically diverse and has considerable potential as an oilseed crop.[6-8] With the current interest in biofuels and bioindustrial feedstocks, *B. carinata* is considered a suitable crop for the production of both ethanol and biodiesel[9-13] or specialty fatty acids (e.g. VLCFAs – this study). In addition, the seed meal, after hydrolysis with digestive proteases such as trypsin, chymotrypsin, and carboxypeptidase, has potential as a source of bioactive peptides (antioxidative, hypocholesterolemic, angiotensin metabolism inhibiting).[14] *Brassica carinata* may also be a good candidate for heavy metal phytoremediation.[15,16]

Although many *B. carinata* genotypes show good agronomic potential, seed quality is lacking. For example, fatty acid profiles of 66 accessions investigated by Warwick *et al.*[17] were observed to be high in erucic acid (22:1 Δ13; 30.9–45.7%) with approximately 5.1–11.6% oleic (18:1 Δ9),

13.7–18.9% linoleic (18:2 Δ9,Δ12) and 10.2–16.0% α-linolenic (18:3 Δ9,Δ12,Δ15) acids[17] (Table 1). Furthermore, all germplasm bank accessions examined in this study were found to be high in glucosinolate content with the primary glucosinolate (>95%) being allyl or sinigrin (2-propenyl) (Table 1). Figure 1 shows a comparison in growth phenotype of field-grown *B. napus* with a noticeably bushier *B. carinata*.

Breeding advances and genetic studies of *B. carinata*:

Germplasm selection and development

Although its adaptiveness to western Canada has not been extensively studied, a preliminary agronomic evaluation of *B. carinata* by Getinet *et al.*[4] indicated that many accessions from Ethiopia were very late maturing and, therefore, not well-adapted to western Canada. Seed yields varied greatly and, on average, *B. carinata* yielded less than the *B. napus* check cultivar.[4] In another study, however, the yield of *B. carinata* cv. S67 was not statistically different from *B. napus* cv. Westar.[18] Getinet *et al.*[4] suggested that Ethiopian mustard has good potential to become a new oilseed or protein crop for western Canada if adapted, early maturing strains could be developed. Subsequently, the Saskatoon Research Centre of Agriculture & Agri-Food Canada (AAFC) initiated a breeding program in the mid-1990s to develop early maturing strains.

Early on in the breeding program, a number of breeding lines were identified that showed that selection for earliness

Figure 1. Comparison of *B. napus* with *B. carinata* in field plots.

Table 1. Mean values for seed quality traits in 66 accessions of *Brassica carinata* grown in a field trial at Saskatoon, Saskatchewan in 1998 (modified from Warwick *et al.*[17]).

Trait	Mean	Std. Dev.	Range
Glucosinolates total (μmoles/g whole seed)	119.8	10.5	87.6–138.7
Alkenyl glucosinolates[1]	116.4	10.5	83.2–135.5
Methylthio glucosinolates[2]	0.1	0.1	0.0–0.6
Indole glucosinolates[3]	2.2	0.5	0.8–3.7
Other glucosinolates	1.0	0.3	0.6–2.0
Fatty acids (% (wt/wt) of total fatty acids)			
18:1 - oleic	7.7	1.1	5.1–11.6
18:2 - linoleic	16.1	0.9	13.7–18.9
18:3 - linolenic	13.3	1.1	10.2–16.0
20:1 - eicosenoic	7.6	0.8	6.2–12.0
20:2 - eicosadienoic	1.1	0.1	1.0–1.3
22:1 - erucic	42.1	2.2	30.9–45.7
22:2 – docosadienoic (Δ13,Δ16)	1.7	0.2	1.0–2.4
24:1 - nervonic	2.5	0.2	2.1–3.4
Other fatty acids	1.6	0.2	0.8–2.2
Total saturated fatty acids[4]	6.2	0.3	5.7–8.0

[1]Includes 2-propenyl (78.5–131.7 μmoles/g whole seed), 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl and 2-hydroxy-4-pentenyl glucosinolate; [2] Includes 3-methylthiopropyl, 4-methylthiobutyl and 5-methylthiopentyl glucosinolate; [3]Includes 3-indolylmethyl and 4-hydroxy-3-indolylmethyl glucosinolate; [4]Includes 14:0 (myristic), 16:0 (palmitic), 18:0 (stearic), 20:0 (arachidic), 22:0 (behenic) and 24:0 (lignoceric).

did not necessarily result in reduced seed yield.[18,19] Equal seed bulks from each of five selected early-to-mature (ETM) populations were tested in replicated multi-location full plot yield trials against their respective unselected base populations. ETM populations were selected for one generation using modified pedigree selection in single-row replicated nurseries. Although days to flower did not vary by more than three days, days to mature varied from zero to six days between ETM and unselected base populations. Also, two of the five ETM populations yielded significantly more than their respective unselected base populations and none of the ETMs yielded less than the corresponding unselected base population. Clearly, high yielding, adapted ETM strains of Ethiopian mustard could be developed for production in western Canada.

After approximately 10 years of breeding for earliness, several promising strains have been developed from these initial populations. On average, selected progenies mature 5–7 days later than most *B. napus* canola; in contrast, the unselected base populations matured 10–14 days later than most *B. napus* canola cultivars in western Canada under semi-arid growing conditions. This is a major achievement since it was done without reducing seed yield relative to the unselected Ethiopian mustard base populations. A subset from a large multi-location and multi-year trial clearly show the progress made in developing early maturing, high yielding strains. The experimental design was a split plot with four species (*Brassica carinata, B. juncea, B. napus* and *B. rapa)*, randomly assigned to main plots in a randomized block design; within each species, five genotypes were randomly assigned to subplots. All data were recorded on a plot basis. Trial data were taken from Saskatoon, Scott and Watrous, Saskatchewan in 2007. Although *B. juncea* yielded more than *B. carinata, B. napus* or *B. rapa*, *B. carinata* yielded more than *B. napus* and *B. rapa* at two of the three locations (Table 2). *Brassica rapa* was the earliest to mature at all three locations, while *B. juncea* and *B. carinata* were the latest to mature. On average, the seed oil content of the five *B. carinata* entries was lower than the other species but its meal protein content was higher.

In addition to early maturity, breeding lines with very high protein content (>35% on a whole seed basis), relatively large seed size (1000-seed weight >3 g), low fiber content, and both low and high erucic acid contents have been developed. The low erucic acid phenotype was first introgressed into the species through interspecific transfer from *B. juncea*.[20] Crosses were made between *B. carinata* cv. S-67 and *B. juncea* Zem 2330, and using *B. carinata* cv. Dodolla as the recurrent backcross parent.[20] Zem 2330 is a zero erucic acid *B. juncea* line derived from the Australian zero erucic acid strain Zem 1.[21] It is interesting to note that zero erucic acid plants had higher levels of linoleic and linolenic acid and lower oleic acid contents than ZEM 2330 or AC Elect (*B. napus*) suggesting that the oleoyl desaturation pathway in Ethiopian mustard is much stronger than in *B. juncea* and *B. napus*.

Seed from this program formed the basis of the low erucic acid program at Agriculture and Agri-Food Canada. Unfortunately, the plant phenotype, even after six backcrosses to cv. Dodolla, was quite poor. Plants were typically a lighter green color and often did not exhibit full fertility (although this was not confirmed through pollen

Table 2. Yield, days-to-maturity, oil and protein content comparison for seeds of various *Brassicaceae*.

	Yield* (kg/ha)			DTM**			Oil (% of seed dry weight)			Protein (% of seed dry weight)		
Location →	Saskatoon	Scott	Watrous	Saskatoon	Scott	Watrous	Saskatoon	Scott	Watrous	Saskatoon	Scott	Watrous
Species												
B. carinata	2104	1262	2987	92	88	95	37.8	35	36.9	28.7	31.6	31.8
B. juncea	2933	2182	3435	89	87	99	47.7	45.9	47.5	23	23.8	25
B. napus	1888	1821	1790	88	87	90	41.9	40.2	40.4	26	27.8	29.2
B. rapa	1640	1390	1697	75	81	72	40.2	39.8	39.5	26.3	26.6	28
L.S.D	282	206	392	2	2	4	1.9	1.4	1.8	1.4	1.3	1.6

*kg/ha
**Days to Maturity.

viability tests). The low erucic acid trait was transferred from *B. juncea* (genomes A & B) without the corresponding transfer from the C genome (from either *B. oleracea* or *B. napus*) and, therefore, was probably a substitution of all or part of a chromosome, or resulted from a cross-over between the A and C genomes. The decision was therefore made to cross BC_6F_2 Dodolla to *B. napus* and then backcross to *B. carinata*. This new source of low erucic acid *B. carinata* germplasm is phenotypically superior to the original and forms the basis of strains combining high seed oil content with low erucic acid content. Low erucic acid strains (<3% erucic) developed by the program have C_{18} fatty acid profiles of (approx.) 31% oleic: 33% linoleic: 27% linolenic (data not shown).

Although the seed oil content of most Ethiopian accessions were found to be 8 to 15% lower than in *B. napus* cv. AC Excel, two lines with seed oil contents above 42% have been developed. Also, because of its large seed size, the protein content is generally higher and the crude fiber content is lower in *B. carinata* than in *B. napus* canola. Considerable variation has also been observed for plant phenotype, leaf form and color, pod architecture, petal color, and shattering resistance.

While pedigree selection is the dominant breeding method used in the improvement of *B. carinata* at AAFC, Saskatoon, SK, a modification of this method best describes our breeding program. Open-pollinated plants within selected rows or small plots are typically selected and advanced. In other words, individuals from within selected progeny rows are threshed individually and advanced to the next generation, following quality analysis. The use of mass selection for disease resistance, single-seed descent and microspore culture has also been used routinely.

Molecular markers, heterosis/diversity and outcrossing

Original morphological analysis suggests that a high amount of variability exists for both growing period and yield traits with moderate variability for oil, glucosinolate and protein content among Ethiopian *B. carinata* accessions.[7] Genetic diversity analysis utilizing both AFLP (amplified restriction fragment length polymorphism) and RAPD (random amplification of polymorphic DNA) techniques display high levels of variation within lines collected in Ethiopia, yet no geographical clustering was apparent.[22,23] Such results suggest that similar selection pressures were present within the entire region. Significant variation was also present between lines from other countries including Pakistan, Spain and Zambia.[22] AFLP and ISSR-PCR (Inter-simple sequence repeat polymerase chain reaction) analyses of lines that have undergone selection for agronomic and quality traits display a substantial loss of genetic diversity indicating that variation is lost rapidly as traits are fixed within *B. carinata*.[8] Interestingly, high levels of genetic diversity were not significantly correlated with heterosis, with phenotypic variation being a much better predictor of heterotic response within *B. carinata* lines.[23] Work by Warwick *et al.*[17] showed that *B. carinata* was less genetically diverse than either the *B. nigra* or *B. juncea* accessions evaluated in the same study. However, the observed variation for some agronomic and quality traits among the 66 *B. carinata* accessions was greater than that previously reported by Getinet *et al.*[4] In addition, AFLP analysis clearly demonstrated the utility of this method to detect genetic diversity. Knowledge of genetic distances of potential parents provides useful information for more efficient parental selection and ultimately, for cultivar development. Such results agree with previous studies carried out in *B. napus* and *B. juncea*.

Outcrossing between *B. carinata* and canola or *B. juncea* is of concern if *B. carinata* is to be used as a molecular farming platform. Successful progeny between *B. carinata* and the related species *B. juncea* and *B. napus* has been reported,[24-25] but there is no published information on large field-scale studies. However, recently a large field-scale study of hybridization between *B. carinata* and *B. napus* was undertaken in 2007 by AAFC in Saskatoon, SK. Results indicated extremely low frequencies of hybridization (<0.002%) between *B. carinata* x *B. napus*; hybridization was detectable in the *B. carinata* field up to 65 m from the pollen source (Dr Ginette Séguin-Swartz, AAFC, pers. comm.). Geographic isolation would minimize the chance of crossing between the three species. However, this cannot be assumed since *B. juncea* and *B. carinata* are adapted to the warmer, drought-prone areas of western Canada and *B. napus* is often grown under irrigation in these areas. The possibility exists that *B. juncea* can act as a bridge to transfer genes from *B. napus* to

B. carinata. Crosses between *B. juncea* and *B. napus* have been reported[24,25] and a recent field-scale study has indicated a low, but detectable level of hybridization (<0.01%) from *B. napus* to *B. juncea* (Ginette Séguin-Swartz *et al.*, unpublished). The study also indicated that most F_1 hybrids had low pollen fertility (<19% viable pollen) and that a few hybrid plants could set seed. Whether such fertile hybrids could hybridize with *B. carinata* under field conditions remains to be determined. Clearly, further studies are required to fully determine the level of outcrossing between these species. In summation, the accidental outcrossing between *B. carinata* and either *B. napus* or *B. juncea* under field conditions is possible, but not likely.

Transformation technologies for *Brassica carinata*

Among oilseed crop species are several members of the *Brassicaceae* which are a source of seed oil for food and industrial uses. While conventional plant breeding has contributed significantly to improvements in *Brassica* crop species, additional improvement is limited by the availability of germplasm with the desired characteristics.

To realize the potential of *B. carinata* as a platform crop for delivery of biofuels, bioindustrial oil feedstocks, edible oils, and for exploitation in molecular farming and phytoremediation, it will be necessary to introduce genes for a variety of characteristics by the application of plant transformation technology. There is little doubt that the use of crops such as *B. carinata* as a source of industrial feedstocks for biofuel and other uses will involve transgenics.[26] This approach has contributed significantly to the improvement of *Brassica* species in general.[27-29]

Consequently, attention has been focused on the introduction of transgenes to improve such features as resistance to biotic and abiotic stress or in modification of seed oil composition, the latter being the focus of the practical examples to follow.

By the transgene approach, desired genes from any source can be incorporated into plants and their expression and stability evaluated. This is achieved by the process of plant transformation where single or multiple genes are introduced into plant cells which are then manipulated to regenerate whole plants. This technology dates back to the early 1980s when the first transgenic plants were produced using *Agrobacterium tumefaciens* (a soil borne bacterium) as a vector to ferry foreign genes into plant cells.[30] There are now a variety of methods employed for gene introduction into plant cells (see review by Newell[31]) but *A. tumefaciens* remains the most frequently used.[30] Transformation and the recovery of transgenic plants are well established in *Brassica* species.[27-29,32] Successful plant transformation relies on a number of factors including the type of vector, cell compatibility with the vector, method of selection of transformed cells and ease of plant regeneration from those cells bearing the foreign genes.

Genetic transformation of *Brassica* in general, has been reviewed previously[27] and since the present review is focused on transformation of *B. carinata*, only passing reference will be made to the pre-1996 literature.

Most transformation systems for *B. carinata* rely on *Agrobacterium*-mediated infection of explants followed by *de novo* shoot regeneration.[32-34] The efficiency of transformation varies with explant type and the selection method. When young stem explants from 7 accessions of *B. carinata* were screened, transformation efficiency was only 1.5% as revealed by Southern blot, kanamycin selection and F_1 segregation patterns.[34] Efficiencies of 30–50% were achieved with cotyledonary petiole explants selected on kanamycin, but only 1–2% when L-phosphinothricine (L-PPT) was the selection agent.[34] Figure 2 shows transgenic *B. carinata* lines at various stages of development in the growth chamber; transgenics were generated by *Agrobacterium*-mediated infection of explants according to the protocol of Babic *et al.*[34] followed by *de novo* shoot regeneration, transfer to soil and growth to the flowering stage at which time plants were bagged to allow 'selfing' of seed. Using a similar protocol with kanamycin selection, Chaudhray *et al.*[33] reported a transformation efficiency of 22%. Plant regeneration from explants of this species appears to be very efficient.[34] However, there is still a need to improve the consistency of transformation efficiency which is influenced by a number of factors including, for example, co-cultivation pH and co-cultivation period.[32,35]

The *in planta* method of *Agrobacterium*-mediated transformation[36,37] has been used in *B. carinata* with a reported efficiency of 1.9%.[38] This is a surprisingly high rate given

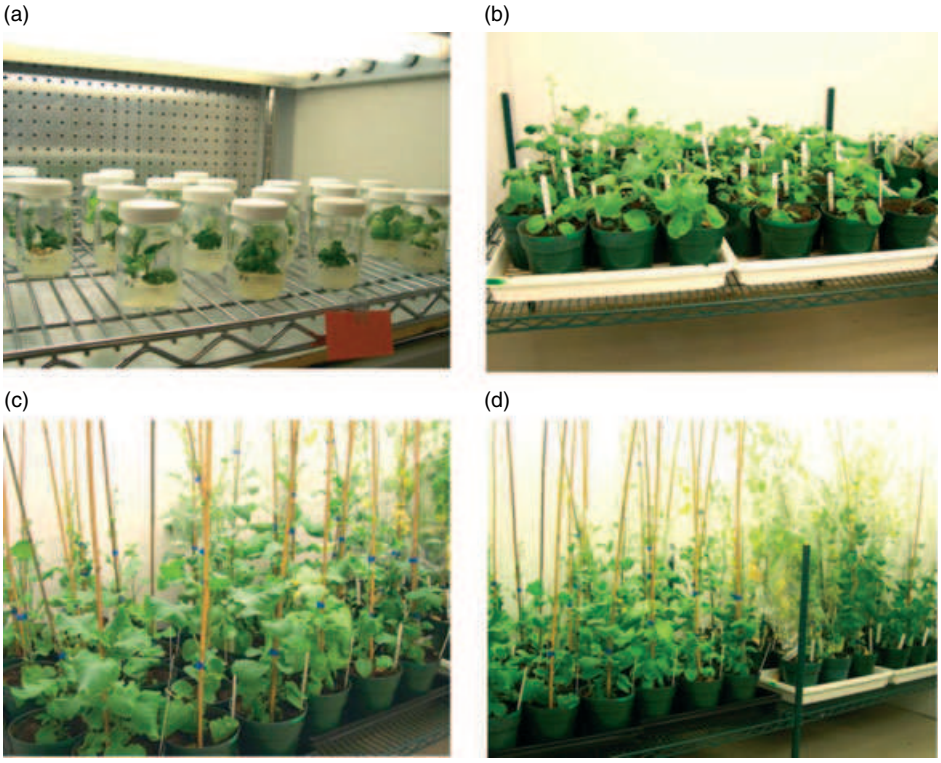

Figure 2. Transgenic *B. carinata* lines (produced using the method of Babic *et al*.[34]) in the growth chamber at various stages of development: (a) Late tissue culture stage-plantlets on shoot-promoting medium; (b) plants transferred to soil in pots; (c) developing transgenic plants at bolting stage; (d) fully bolted and flowering transgenic plants bagged for self-pollination ('selfing').

that the method relies on infiltration of *Agrobacterium* into floral meristems, insertion of the T-DNA 'cargo' into germ line cells and screening of subsequently produced seeds for expression of the introduced gene. It is not clear whether the floral meristem is differentially susceptible to *Agrobacterium*, compared to a vegetative meristem and the exact mechanism of infection is not understood.[39,40] However, this method is very successful for transformation of *Arabidopsis* and is being used increasingly for *Brassica* transformation.[40-42] Refinement of this protocol for *B. carinata* transformation is desirable as this will avoid somaclonal variation which frequently occurs during *de novo* plant regeneration from cultured explants. By low temperature induction of early bolting and flowering and removal of flowers emerging after vacuum infiltration, Wang *et al*.[41] obtained transformation rates of 50–60% with *B. napus*. With *B. rapa* ssp. chinensis, Xu *et al*.[43] obtained transformation frequencies of 4 to 23 events per 10 000 seeds, depending on the location of the siliques on the inflorescence.

The biolistic or particle bombardment method of transformation, wherein DNA-coated particles are shot into plant tissues, is a highly effective method.[44-46] With this method, vectors are not required and it is species and genotype independent. Although fertile transgenic plants were recovered by particle bombarding isolated microspores of *B. napus*,[47] this has not been extended to *B. carinata*. Particle bombardment is the most reliable approach for the transformation of plastids.[48] In *Brassica* species, stable chloroplast transformation has been reported for *B. oleracea*[49] but with *B. napus*, transformants were unstable as homoplasmy was not achieved.[50] Since very large amounts of heterologous protein can be produced in the chloroplast,[51] procedures for stable plastid transfomation of *B. carinata* would be a distinct advantage, especially for molecular farming of bioactive peptides and vaccines as there would be no pollen flow from transgenics using this method.

Other methods of *Brassica* transformation: PEG-mediated DNA uptake, microinjection and electroporation, as

reviewed by Poulsen,[27] have been somewhat de-emphasized in recent years in favor of *Agrobacterium*-mediated and particle bombardment methods and there are no reports of their use in the transformation of *Brassica carinata*. Techniques such as PEG-mediated DNA uptake and electroporation, however, are useful for introducing large pieces of DNA into plant cells with the advantage that both plastid and nuclear transformation can be achieved at the same time.[52]

While *Agrobacterium tumefaciens*-mediated transformation is the most frequently used method to produce transgenics in this species, there is still a need to improve the efficiency of transformation and recovery of transgenic plants. This can be achieved by careful explant selection and by improvement in shoot regeneration frequency in a genotype independent manner. If the *in planta* method of transformation can be made more efficient, this would greatly facilitate the recovery of transgenics. *Agrobacterium rhizogenes* has been used as a vector for the transformation of *Brassica* species.[53,54] The phenotypic aberrations and low fertility of the transgenics make this vector unsuitable for transformation where fertile seeds are required. The disarmed *A. rhizogenes* vector may prove useful as it was 3.5-fold more efficient in transforming soybean explants compared to *A. tumefaciens* and plants were phenotypically normal and fertile.[55] The versatility of particle bombardment-mediated direct DNA uptake[46] should be useful for transformation of *B. carinata* as it facilitates transformation of both plastid and nuclear genomes and has been used to incorporate insect resistance into *B. napus*.[56]

Isolated microspore culture systems of *B. napus* have been used for transformation and the recovery of transgenic plants.[47,57] Although this system is currently inefficient, it has several advantages over the regular adventitious shoot regeneration system. The introduced gene can be readily fixed in the homozygous state by chromosome doubling with colchicine. Refinement of this protocol for *B. carinata* transformation is desirable as this will avoid culture-induced changes which frequently occur during *de novo* plant regeneration from cultured explants. Regeneration is by embryo formation and these embryos usually germinate into plantlets without the need for transplanting to rooting media. Embryos can be induced from several genotypes of *B. carinata*[58,59] and this could be a viable system for transformation and regeneration of transgenic *B. carinata* plants.

In general, future advances in the transformation of *Brassica* oilseed species will depend on improvements in both transformation and plant regeneration efficiencies. The introduction of single gene traits is likely to be replaced by multi-gene traits involving several genes, artificial chromosomes and co-transformations. The incorporation of multiple traits will require modification of current methods of transformation. For example, polyethylene glycol (PEG) is now commonly used for the introduction of artificial chromosomes into plant cells (protoplasts) but the efficiency needs to be improved. Although particle bombardment is regarded as the more efficient method for introducing multiple genes into plant cells,[46] up to 7 genes were introduced into *B. napus* hypocotyl explants using *Agrobacterium tumefaciens*.[60] In this case, 73 to 85% of the regenerated plants expressed all 7 genes. Therefore, the transformation method *per se* may not be a limiting factor for multiple gene transfer into plant cells. Gene 'stacking' or 'pyramiding' will most certainly be required to confer tolerance to complex traits such as biotic and abiotic stresses.[61] To fully exploit the potential of *B. carinata* as a platform crop, it will be essential to develop an efficient system for the transfer of single or large sets of genes into the appropriate explant and a system for the recovery of a large enough population of transgenics from which plants with the desired traits can be selected. Below we describe the successful use of a two-gene stack in manipulation of the metabolic pathway for producing higher erucic acid in *B. carinata* oil.

Case studies in the application of genetic engineering to enhance seed oil profiles in *B. carinata*

Very long chain fatty acids: industrial and pharma-nutraceutical interest and applications

Very long chain fatty acids (VLCFA) are those that contain more than 18 carbon atoms. They are common components of seed oils and plant waxes in a number of plant families including the *Cruciferaceae*, *Limnanthaceae*, *Simmondsiaceae* and *Tropaeolaceae*.[62] A strategic goal of our research is to modify seed oil composition in the *Brassicaceae* to increase the proportion of VLCFAs. While we have HEAR (high erucic acid rapeseed) *B. napus*

cultivars in existence in western Canada and winter cultivars in Europe, 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 prairies. As detailed previously, *B. carinata* is easily transformed at a very high efficiency,[34] is highly disease-resistant (e.g. blackleg), and is drought-tolerant, amenable to growth in hotter, drier regions. The new breeding lines of *B. carinata* with a higher oil and higher glucosinolate content will provide excellent germplasm for production of high erucic and other industrial oils. The idea of developing *B. carinata* with high allyl glucosinolate meal is to either use it directly as a biopesticide (e.g. Peacock Industries EcoBran™). For this application, some processing is required and myrosinase is added back to ensure that the allyl isothiocyanate is released upon the addition of water). Additionally the meal can be processed with a solvent wash to remove the glucosinolates and the remaining low fiber meal used as fish feed.

Erucic acid (*cis*-docosa-13-enoic acid, 22:1 Δ13) (Fig. 3) is the major VLCFA in the seed oil from HEAR *B. napus* cultivars, accounting for 45–55% of the total fatty acids.[63] Only the seed of *Tropaeolum majus* (garden nasturtium) contains more than 75% erucic acid and trierucin as its major triacylglycerol (TAG).[64] HEAR cultivars are of high interest for industrial purposes because 22:1 is a valuable feedstock with more than 1000 potential or patented industrial applications.[65]

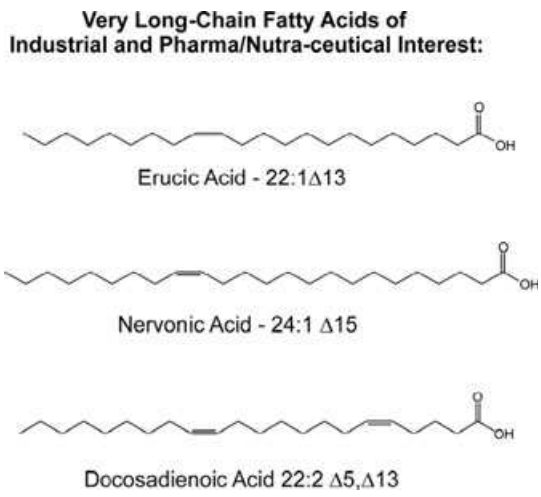

Figure 3. Nomenclature and structures of target VLCFAs discussed in this study.

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, e.g. in lubricants, detergents, film processing agents and coatings, as well as in cosmetics and pharmaceuticals.[66-70] Studies have confirmed that high erucic oil and its derivatives have a higher energy potential than low erucic oil.[71] Compared to low erucic *Brassica* oils, high erucic oils are more suitable for biodiesel production because their iodine value is lower and within the European Union specifications.[72] US industry uses 18 million kg of high erucic acid oil annually, mostly from imports, but (historically), supplies are limited. Therefore, a large overall market potential exists for expansion and development of new annually-renewable domestic sources of erucic acid, principally for export.[73]

Nervonic acid, (*cis*-tetracos-15-enoic acid; 24:1 Δ15) (Fig. 3) is another strategic VLCFA; it also has the colloquial name of selacholeic acid. Nervonic acid exists in nature as an elongation product of oleic acid (18:1 Δ9). The immediate precursor to nervonic acid, erucic acid (22:1Δ13), has a substantially different distribution in plants and animals compared to nervonic acid. Nervonic acid is found in the triacylglycerols in the seeds of only a few known plants: *Lunaria* spp. (money plant), borage, hemp, *Acer truncatum* (purpleblow maple), *Tropaeolum speciosum* (flame flower) and *Cardamine graeca* (bittercress), in all cases predominantly at the *sn*-1 and *sn*-3 positions on the glycerol backbone.[74] In contrast, nervonic acid is generally not found in appreciable quantities in the triglycerides of animals. However, nervonic acid is widely distributed in the sphingolipid fractions of the tissues of vertebrate animals, where it is bound via an amide bond to a sphingosine base.[75] 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 fraction of myelinated nerve fibers.[76] In contrast, erucoyl-sphingolipid is largely absent as from animal tissues.[77]

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 function of myelin in brain and nerve

tissues.[78] Specifically, the proposal suggested that dietary supplementation with nervonic acid might be beneficial, for neurological development/function, in: (1) individuals with genetic disorders of lipid metabolism specifically associated with peroxisomes (adrenoleukodystrophy, Zellweger's syndrome, others); (2) individuals with multiple sclerosis and other nervous disorders such as Parkinson's disease;[79] and (3) human infants, particularly premature infants, receiving infant formula as a source of nutrition. This proposal encouraged the development of a refined, nervonic acid-enriched plant oil (Croda, *Lunaria biennis*-derived) for feeding trials on humans and animals.[79] *Lunaria* oil contains about 45% erucic acid and 20% nervonic acid. In spite of this development, nutritional studies on nervonic acid are limited by the lack of availability of a nervonic-acid-rich oil that has minimal amounts of erucic acid (William Bettger, pers. comm.).

Recent developments in the genetics and metabolism of very long chain fatty acids,[80-82] in sphingolipids as signaling molecules,[83,84] and in the roles of sphingolipids carrying VLCFAs as structural components of the lipid rafts present in the outer leaflets of cell plasma membranes[85,86] suggest that dietary nervonic acid has the potential to have distinct physiological effects, depending on the level in the diet. At low levels of 0.1% or less in the diet, nervonic acid could compete with 24:0 (and to some extent 22:0) for incorporation into sphingolipids in extra-neural tissues. This could result in subtle but predictable changes to the functions of a variety of tissues in the body via structural alterations in plasma membranes and in sphingolipid cell-signaling. At higher levels of dietary intake, nervonic acid may modulate gene expression directly by binding to a number of transcription factors, altering whole body lipid and energy metabolism. Under conditions of low erucic acid intake, dietary nervonic acid is predicted to be relatively non-toxic to humans and animals. Nervonic acid is therefore a strong candidate to be further evaluated as a bioactive lipid supplement, similar to arachidonic acid, docosahexaenoic acid and conjugated linoleic acids, for the promotion of human and animal health (e.g. Young and Conquer[87]).

***cis, cis* Docosa- 5, 13 dienoic acid (22:2 Δ5, Δ13)** (Fig. 3). A number of plants produce seed oils enriched in unusual fatty acids with a Δ5 functionality, including species of meadowfoam: *Limnanthes douglasii* and *L. alba*. *Limnanthes* seed oils are enriched in Δ5-eicosenoic acid (20:1 Δ5) and, to a much lesser extent, an unusual diene, Δ5, Δ13-docosadienoic acid (22:2 Δ5, Δ13).[88] Because of their unique double bond positioning, both of these fatty acids are of strategic interest as industrial feedstocks. Its oxidative stability and high content of VLFCAs impart to the seed oil of *Limnanthes* species a number of properties that are desired by the cosmetic, surfactant, and lubricant industries.[89-92] The 20:1 Δ5 component of this oil can also serve as a chemical precursor of compounds such as estolides and δ-lactones that can be used for a wide range of industrial applications, including lubricants and plasticizers.[91, 93] The relatively high price of meadowfoam oil, however, limits its commercial use to primarily cosmetic applications, and as a result, this plant is currently grown only as a niche crop in the Pacific Northwest of the United States. The small but significant proportion of a unique diene, 22:2 Δ5, Δ13 in meadowfoam oil (10–15%) is also of industrial interest.[88] This diene possesses widely spaced, non-conjugated or methylene-interrupted double bonds making it quite stable and not as prone to oxidation. There are niche market applications that have been identified for its use as a feedstock for generating estolides, which can be used to synthesize hydroxy fatty acid feedstocks, and to produce dimer acids, esters and amides for use as lubricants, and slip-promoting anti-block agents in plastic film manufacturing.[90,93]

Some of the many industrial and health-dietary supplement related uses of VLCFA oils are shown in Tables 3 and 4**.**

Biosynthesis of VLCFAs seed oils in the *Brassicaceae*

In oilseeds, VLCFAs are synthesized in the developing cotyledons by a microsomal fatty acid elongation (*FAE*) or 'elongase' complex using acyl-CoA substrates from a cytoplasmic pool maintained by *de novo* lipid biosynthesis in plastids. As shown in Fig. 4, each cycle of fatty acid elongation adds two carbon units to the acyl chain and involves four reactions: first, malonyl-CoA and long chain acyl-CoA are condensed by a 3-ketoacyl-CoA synthase (KCS); the resulting 3-ketoacyl-CoA is then reduced by the action of a 3-ketoacyl-CoA reductase resulting in the synthesis of a 3-hydroxyacyl-CoA. Subsequently 3-hydroxyacyl-CoA is dehydrated to a

Table 3. Examples of industrial applications of high erucic or high nervonic oils.

Industrial Applications
Viscoelastic surfactants and high molecular weight anionic surfactants
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 for film processing
Enhanced Oil Recovery Surfactants
Paving bed polymers
High temperature lubricants for intact VLC oil (TAG)

Table 4. Examples of potential applications of high nervonic oils as nutritional supplements and in the treatment/diagnosis of various disease states with respect to nervonic acid levels.

Nutritional Applications
In infants or young children during the key 'myelinating stage' of neuro development (up to age 5) e.g. baby food and infant formula supplementation
Pre-term babies, where the infant no longer benefits from maternal nutrition
Supplement for women who intend to become pregnant, are pregnant or lactating
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 is available in non-fat dairy and whey products
Nervonic Acid and Disease States
Demyelinating diseases such as Multiple Sclerosis (MS) and Adrenoleukodystrophy (ALD)
Parkensonian tremors
Marked reduction in nervonate sphingolipids in post-mortem analyses of MS and ALD patients
Defects in microsomal biosynthesis of very-long chain fatty acids including nervonate in 'jumpy' and 'quaking' mice model systems is accompanied by impaired myelination
Schizophrenia- nervonic acid is very low in these patients
HIV- Nervonic acid dose-dependently inhibits HIV-1 reverse transcriptase activity
Nervonic acid deficiencies associated with various other neurological disorders/conditions

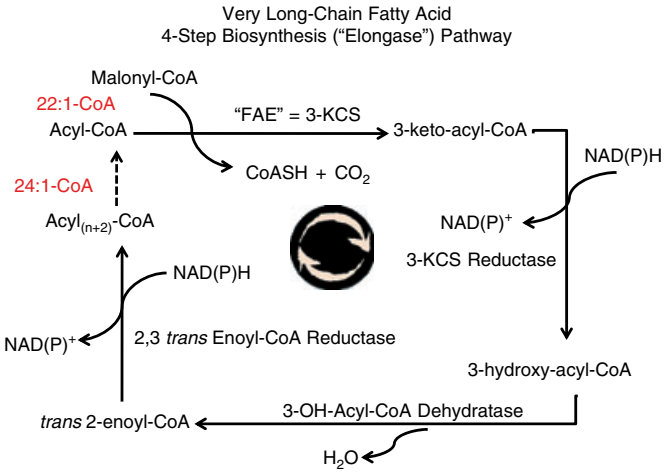

Figure 4. The 4-enzyme microsomal elongase complex responsible for VLCFA biosynthesis in oilseed cotyledons. The key first condensing enzyme, 3-ketoacyl-CoA synthase (**KCS**) was often designated as 'FAE- fatty acid elongase' in the older literature. The steps for each 'turn' of the elongation cycle are shown with the example being the elongation of erucoyl (22:1)-CoA to nervonoyl (24:1)-CoA.

2-enoyl-CoA, which is then reduced by second reductase to form the elongated acyl-CoA. Over the past 15 years, progress in understanding VLCFA biosynthesis has been achieved by cloning *KCS* genes from different plants and performing functional expression studies.[62,69,70, 94-101] These and other studies have provided evidence that KCS is the rate-limiting enzyme for seed VLCFA production[101,62] and that it is the substrate specificity of the KCS enzyme which determines the chain length produced. Due to the membrane-bound nature of the KCS protein, our knowledge of the properties, and regulation of this enzyme are still limited.[101]

The proposed biosynthetic pathway for 20:1Δ5 in *Limnanthes* species involves three steps:[103,104] (1) a flux of palmitic acid (16:0) from the plastid to the ER, followed by (2) microsomal elongation of 16:0-CoA to 18:0-CoA and then 20:0-CoA and finally (3) desaturation of 20:0-CoA at the Δ5 position catalyzed by an enzyme designated Des5, to yield 20:1 Δ5-CoA which is then incorporated into glycerolipids. The proposed pathway for 22:2 Δ5, Δ13 biosynthesis is thought to involve a further desaturation of 22:1 Δ13-CoA at the Δ5 position, also catalyzed by Des5 (Fig. 5).

To our knowledge, all of the VLCFA biosyntheses involve acyl-CoAs as the immediate primers.[97,99] There is no evidence

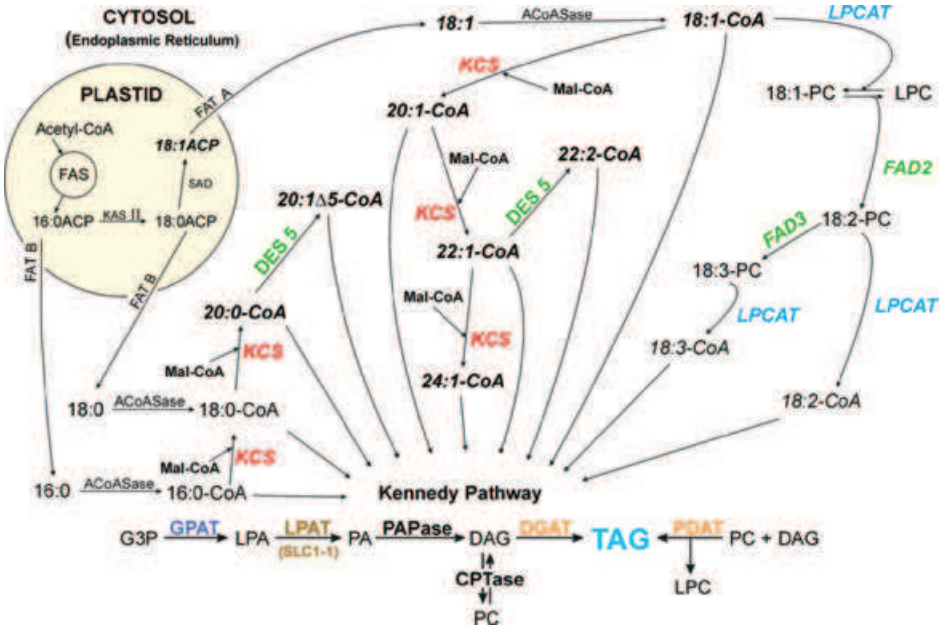

Figure 5. Diagram showing the intersecting biochemical pathways for fatty acid and glycerolipid biosynthesis in oilseed cotyledons of the *Brassicaceae* and key enzymes discussed herein: KCSs are the elongase enzymes, 3-keto-acyl-CoA synthases (EC 2.3.1.85), which catalyze the first step in a four-enzyme Fatty Acid Elongasse complex, which produces the VLCFAs; FAD2 and FAD3 are the 18:1 (EC 1.3.1.35), and 18:2 (no EC no assigned to date) desaturases creating 18:2 and 18:3, respectively; Des5 is the Δ5 destaurase which creates 22:2; CTPase, CDP-choline:diacylglycerol cholinephosphotransferase (EC 2.7.8.2); DGAT, diacylglycerol acyltransferase (EC 3.2.1.20); GPAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); LPAT, *lyso*-phosphatidic acid acyltransferase (EC 2.3.1.51); LPCAT, *lyso*-phosphatidylcholine acyltransferase (EC 2.3.1.23); PAPase, phosphatidic acid phosphataste (EC 3.1.3.4); PDAT, acyl-CoA-independent phosphatidylcholine (EC 2.3.1.158); SLC1-1, *Saccharomyces cereviseae* LPAT.

that any of these reactions occur while the acyl groups are esterified to a glycerol backbone, as is the case for example, in the synthesis of 18:2 Δ9, Δ12 and 18:3 Δ9, Δ12 Δ15 which are produced by successive desaturations of 18:1 Δ9, by the enzymes FAD2 and FAD3, respectively, while the oleoyl moiety is esterified to the *sn*-2 position of phosphatidylcholine.[105] Interestingly, both the polyunsaturated fatty acid biosynthesis pathway and the VLCFA pathway involve oleic acid (18:1 Δ9) as the original acyl chain donor. Thus, in the developing oilseed, there is often a competition between the two pathways- elongation and desaturation- for oleoyl moieties.

The interaction of these various VLCFA biosynthesis pathways, and the associated enzymes and encoding genes which we have manipulated through metabolic engineering in *B. carinata* are presented in Fig. 5, and will be discussed below.

Case studies: Metabolic engineering of *B. carinata* oils for enhanced proportions of strategic VLCFAs and oil content

High erucic acid oil

A strategic goal of our research is to modify seed oil composition to increase the proportion of erucic acid (22:1Δ13) in *Brassicaceae*.

In order to maximize the proportion of erucic acid in *B. carinata* we performed transformation experiments utilizing two plant KCSs with different substrate preferences (*Arabidopsis KCS* and nasturtium *KCS*; Figs 6(a) and 6(b)). The former prefers to elongate 18:1 to 20:1, while the latter prefers to elongate 20:1 to 22:1. When expressed in tandem in *B. carinata*, we found that in transgenic lines the carrying

the *Arabidopis KCS* + nasturtium *KCS*, erucic acid increased from 36% (wt/wt) of total fatty acids in non-transformed wild type to as high as 47% (wt/wt) in the T_3 generation (Table 5). This represents a net increase of 29% in erucic acid content. Furthermore, oil contents in the transgenic lines were 100–114% of the non-transformed wild type (nt-WT) C90-1163 controls (Mietkiewska and Taylor, unpublished).

Examining other sources of strategic *KCS* genes we selected *Crambe abyssinica* (Fig. 6(c)). The seed oil of *C. abyssinica* is distinct from other *Brassicaceaee* because of its very high proportion of erucic acid which is up to 55% in native accessions.[66] Therefore, we isolated and functionally characterized a *C. abyssinica KCS* homolog and expressed the *CrKCS* under the control of the napin promoter in *B. carinata*.[70] The results of the analyses of seed oil of 9 best *B. carinata CrKCS* lines selected from the T_3 generation showed that erucic acid was increased to as high as 52.7%, while the proportion of total VLCFAs (C_{20} or greater) rose from 54.9% in the wild-type control to as high as 66.1% in the best transgenic line. Most of this increase can be attributed to the erucic acid increase induced by expression of the *CrKCS gene*. In confined transgenic field trials conducted at two locations in Saskatchewan in 2007, the relative increase in the proportion of erucic acid in the *CrKCS* transgenic

Table 5. Erucic acid (22:1) proportions (% wt/wt) in the oil from mature seeds of non-transformed *Brassica carinata* wild type control line C90-1163 (NT-Wild Type) and T_3 mature seeds of *Brassica carinata* C90-1163 transgenic lines carrying both the *Arabidopsis FAE1* and nasturtium *FAE* genes (NFPC + AFAE) or carrying the *Crambe FAE* + RNAi –silenced *B car FAD2* genes (XS). Values are reported as % 22:1 (wt/wt) of the total fatty acids ±S.D. and are the average of 3 determinations.

Line	22:1 % (wt/wt) of Total Fatty Acids
NT-Wild Type	36.7 ± 0.3
NFPC+AFAE[a]	47.9 ± 0.7
XS[b]	58.0 ± 0.7
Data from	[a]Mietkiewska et al.[62] [b]Mietkiewska et al.[106]

Figure 6. (a) *Arabidopsis thaliana* (Thalecress) (b) *Tropaeolum majus* (Garden nasturtium), (c) *Crambe abyssinica* (Abyssinian mustard)- 3 sources of *KCS* genes expressed in *B. carinata* to enhance erucic acid content. (d) *Lunaria annua* (Moneyplant) (e) *Cardamine graeca* (Bittercress)- 2 sources of *KCS* genes expressed in *B carinata* to improve nervonic acid; (f) *Limnanthes alba* (Meadowfoam)-source of *Des5 desaturase* gene to allow production of 22:2 in *B. carinata*.

lines was consistently observed, and in fact, a few percent better than the greenhouse studies (Taylor, unpublished).

Because of the apparent competition of the 18:1 desaturation (*FADs 2* & *3*) and 18:1 elongation (*KCSs*) pathways for oleoyl primers (Fig. 5), we reasoned that the desaturation pathway was perhaps another key step limiting erucic acid biosynthesis and deposition in *B. carinata*. Accordingly, we used both anti-sense and co-suppression technologies to partially silence the *FAD2* gene which converts 18:1 to 18:2, with the effect that the proportions of VLCFAs and erucic acid in particular, were increased due to reduced flux of oleate into the desaturation pathway and concomitant flux of oleoyl moieties into the elongation pathway.[105] Specifically, co-suppressed *FAD2 B. carinata* lines exhibited, on a relative basis, 3–18% decreases in 18:2, 22–49% decreases in 18:3 and significantly increased proportions of 18:1 (36–99%), 22:1 (12–27%), and VLCFAs (6–15%). In comparison, transgenic *B. carinata* lines with antisense-repressed *FAD2* exhibited, on a relative basis, decreases in 18:2 and 18:3 of 9–39% and 33–48%, respectively, and increases in 18:1 (54–130%), 22:1 (5–19%) and VLCFAs (6–21%). Thus, the possibility of using these silencing approaches to produce prototypic transgenic germplasm of *B. carinata* with erucoyl-enhanced seed oils was confirmed.

Accordingly, we have found that over-expression of the *KCS* from *Crambe abyssinica* combined with RNAi-*FAD2* silencing/reduction in oleoyl desaturation in *B. carinata* results in an increase in 22:1 in *B carinata* oils to as high as 58% (wt/wt) in the best T_3 line, a net 22% increase compared to the non-transformed controls[106] (Table 5). This confirms the potency of this double gene technology for re-routing metabolism to enhance erucic acid production in *B carinata*.

Ultimately, we wish to further modify these high erucic *B. carinata* phenotypes to produce very high erucic acid lines with more than 70% 22:1. To reach this next plateau will require that we address a limitation of the lipid bioassembly pathway which severely limits the incorporation of significant 22:1 at the *sn*-2 position on the glycerol backbone of TAGs. This key biochemical/metabolic bottleneck is created by the substrate limitation of the *lyso*-phosphatidic acid acyltransferase encoded by the *LPAT* gene indigenous to all members of the *Brassicaceae;* these LPATs cannot effectively acylate the *sn*-2 position of LPA to give dierucoyl PA in the Kennedy pathway (Fig. 5) the result being that TAGs of the *Brassicaceae* contain erucic acid at the *sn*-1 and to a larger extent, the *sn*-3 positions, limiting the erucic acid content to a maximum of about 66% (wt/wt). As our model, since 2006, we have utilized the generally unsubscribed *T. majus* (garden nasturtium) seed as a model for cloning and expressing genes essential to the production of trierucin. We have generated a collection of 20 000 ESTs from a library of subtracted developing nasturtium embryo cDNAs, which we will continue to 'mine' to isolate key genes to effect trierucin synthesis in *B. carinata.* To this end, we recently cloned and characterized an *LPAT2* from nasturtium (*TmLPAT2*) that will catalyze the synthesis of dierucoyl PA in the Kennedy pathway.[107] Current experiments underway will re-transform the *Crambe KCS*+ RNAi *FAD2* dual transgene *B. carinata* line with the *TmLPAT2* to try to produce *B. carinata* prototypes with 70% erucic acid or better. Recently it has been shown that by combining alleles of *B. napus* related to low polyunsaturated oils with the transgenic co-expression of the *L. douglasii LPAT2* and the *BnFAE1* (encoding *BnKCS*), a *B. napus* line with oil containing just over 70% erucic acid was obtained, clearly a breakthrough.[108]

High nervonic acid oil

Our goal was to isolate and characterize strategic new genes for high nervonic acid production in *Brassica* oilseed crops. To this end, we isolated *KCS* genes from *Lunaria annua* (Fig. 6(d)) and from *Cardamine graeca* (Fig. 6(e)) seed oils of which contain significant levels of 24:1. Functional expression of these *KCS*s in *B. carinata* resulted in strong increases in seed oil nervonic acid proportions. KCS enzyme activity assays indicated that upon using ^{14}C-22:1-CoA as substrate, the production of ^{14}C 24:1 from developing seeds of transgenic *B. carinata* was up to 30-fold higher than the low erucoyl-elongation activity exhibited by wild type control plants.[109] The highest nervonic acid level in transgenic *B. carinata* expressing the *Lunaria KCS* reached 30%, compared to 2.8% in wild type plants. (Fig. 7). In addition, the erucic acid proportions in these transgenic lines were considerably lower than that found in native *Lunaria* oil. However, while showing the functional utility of the *Lunaria KCS* in engineering new sources of high nervonate oils in the *Brassicaceae*, the result in terms of functionality of the transgenic oil in

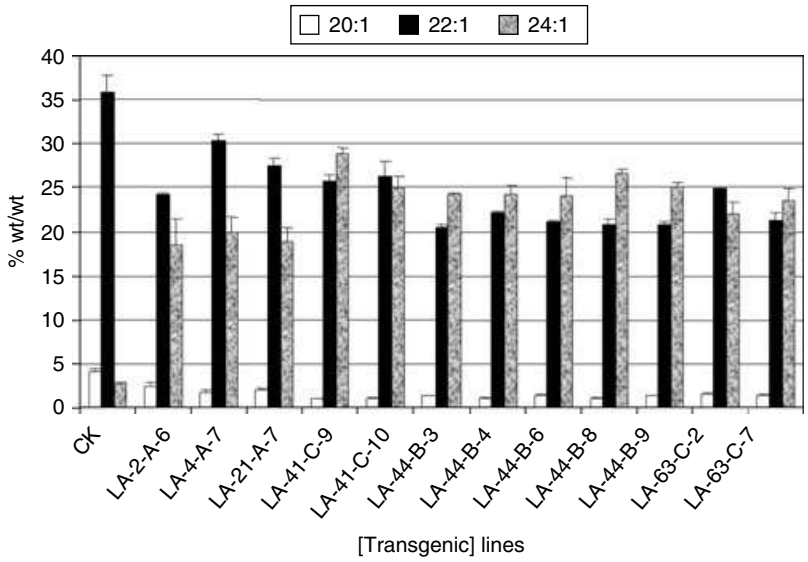

Figure 7. Fatty acid composition of T_3 seed oil of transgenic *B. carinata* transformed with the *Lunaria annua KCS* gene. Proportions of 20:1 (white bars), 22:1 (black bars) and 24:1 (grey marbled bars) in seed oils from empty plasmid-only transgenic control line (CK), and the twelve best *B. carinata* T_3 transgenic lines expressing the *L. annua KCS* gene (LA) under control of the napin promoter. Values are presented as % wt/wt of each fatty acid ±SD. Data from Guo *et al.* [109]

nutra/pharma-related formulations was less than desirable because the proportions of erucic acid in the best T_3 lines were equal to, or greater than, that of nervonic acid;[109] traditionally, high erucate *B. napus* oils are undesirable for human consumption.

In contrast, expression of the *Cardamine KCS* in *B. carinata* had a much better outcome in this regard[110] As shown in Fig. 8, the proportions of 24:1 in the best T_3 transgenic *B. carinata* lines harboring the *Cardamine KCS* gene were as high as 45%, while the erucic acid content was less than 7% in many lines. The best line had a nervonic content of 42.5% while erucic acid was as low as 5.5%. Of equal importance is the fact that the proportions of the healthy fatty acids 18:2 + 18:3 (ω-6 and ω-3, respectively) totaled about 35% (w/w) of the total fatty acids (Fig. 9).

Figure 10 shows a confined transgenic field trial conducted in 2009 of the T_3 generation which indicated that the best performing line, containing only a single insert of the *Cardamine KCS* gene, yielded oil with as much as 45% 24:1 and an erucic acid content below 7%. Given that the 2009 season was one of the poorest growing seasons of recent record in Saskatchewan, the performance of the *Cardamine KCS* transgenic lines was excellent, with the acyl profile remaining stable and with oil contents well within the range of the empty-plasmid controls.

As is the case with erucic acid, the *B. carinata* native LPAT cannot incorporate 24:1 into the *sn*-2 position on the glycerol backbone. Accordingly, future work with this line will involve introducing the nasturtium LPAT (*TmLPAT2*) as described above, only this time, in order to enhance the proportion of nervonate in the middle position of TAGs, and therefore hopefully boost the overall 24:1 proportions considerably.

Despite the limitation in the overall nervonate proportions of the current transgenic oils, they are, at 45% 24:1/<7%22:1, of a purity sufficient to test in a range of animal disease models and in several lucrative industrial applications.[74] The spin-offs for this new oil already show promise.

22:2 Δ5, Δ13 oil

As mentioned above, seeds of *Limnanthes* spp. (meadowfoam; Fig. 6(f)) contain novel VLCFAs of strategic importance for a number of industrial applications, including the monoene 20:1Δ5 and the diene 22:2Δ5, Δ13, the latter being

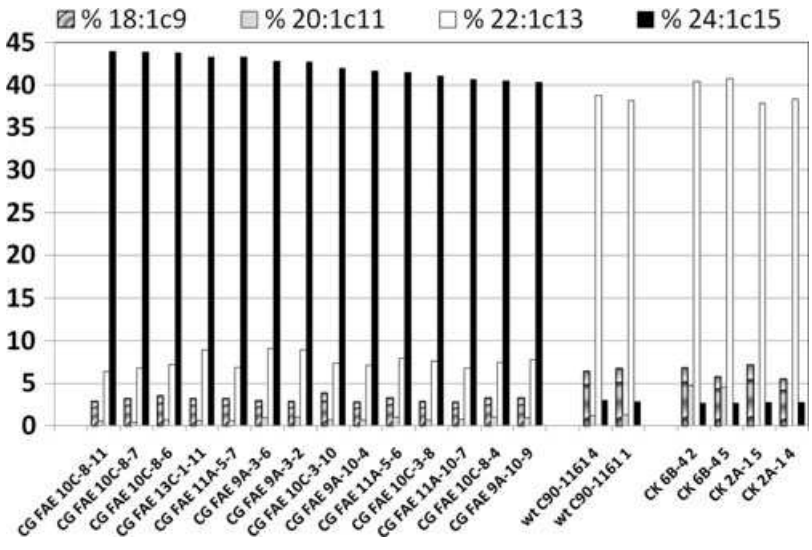

Figure 8. Mono-unsaturated fatty acid composition of T_3 seed oil of transgenic *B. carinata* transformed with the *Cardamine graeca KCS* gene. Proportions of 18:1 (grey marbled bars), 20:1 (grey bars), 22:1 (white bars) and 24:1 (black bars) in seed oils from non-transformed WT (wt C90), empty plasmid-only transgenic control line (CK), and the 14 best *B. carinata* T_3 transgenic lines expressing the *C. graeca KCS* gene (CG) under control of the napin promoter Values are presented as % wt/wt of each fatty acid ± SD.

of our particular interest. Engineering of meadowfoam-type oils in other oilseed crops is desirable for the production of these fatty acids as industrial feedstocks, particularly given the very limited acreage devoted to *Limnanthes* in North America and several less-than-ideal agronomic properties as a crop, one being an oil content of about 22%. 22:2Δ5, Δ13 proportions in *Limnanthes* range from 10–15% (wt/wt), giving this seed a 22:2Δ5, Δ13 content ranging from 2.0–3.6 mg/g DW. A *Limnanthes* seed-specific cDNA (designated *Lim Des5*) encoding a homolog of ubiquitous acyl-coenzyme A desaturases found in animals, fungi and cyanobacteria, was cloned[111] and expressed in *B. carinata*, which resulted in the accumulation of up to 10% 22:2Δ5, Δ13 in the seed oil, which represented about 20% of the total VLCFAs (Table 6).[112] These results demonstrate the utility of *B. carinata* for the production of vegetable oils containing other novel C_{22} fatty acids, and confirm that one of the preferred substrates of the *Lim* Des5 enzyme is erucic acid (22:1Δ13). Importantly, our *B. carinata* Des5 transgenic prototypes with about 30% oil and 10% 22:2, are already approaching the same range for total 22:2 content (3.0 mg/g DW) as native *Limnanthes* seed as cited above, making our transformants a potential competitive source of this unique diene.

Oil content improvements

One of biggest challenges regarding utility of *B. carinata* as an oilseed crop is its oil content which is typically about 10% lower than that in canola *B. napus*. Even given the added average seed weight (yield) advantage that *B. carinata* holds over *B. napus*, enhancing oil content in *B. carinata* will be necessary, either through breeding or via transgenic means, to enhance its chances of improving economic return at the farmgate and thereby becoming a new industrial oilseed platform. There have been significant strides to enhance oil content via breeding as shown in Table 2. Here we demonstrate the utility of transgene technology to improve this trait in breeding lines of *B. carinata*. It should be noted that as in the case for the VLCFA work described above, the lines we have used in this endeavor are much earlier versions of breeding lines than those reported in Table 2; therefore what we are demonstrating here is the advantage of *relative* oil content improvements achieved through transgenic experiments.

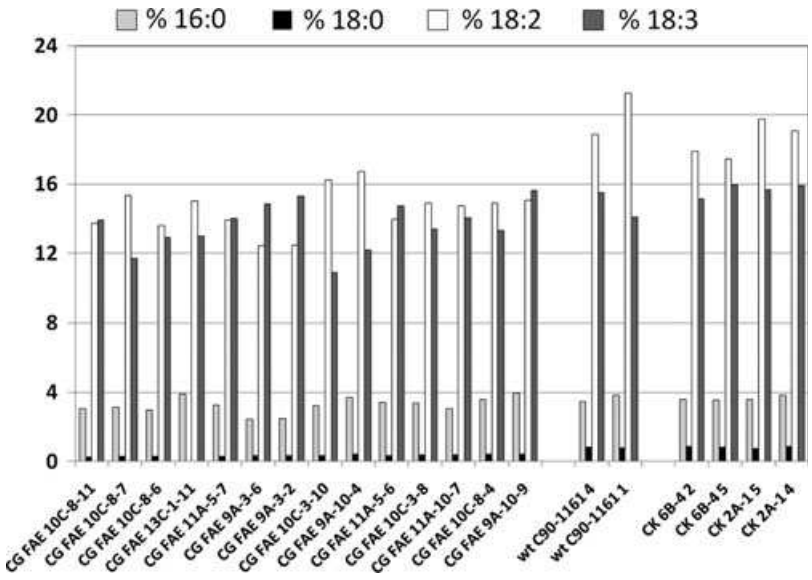

Figure 9. Saturated and polyunsaturated fatty acid composition of T_3 seed oil of transgenic *B. carinata* transformed with the *C. graeca KCS* gene. Proportions of 16:0 (light grey bars), 18:0 (black bars), 18:2 (white bars) and 18:3 (dark grey bars) in seed oils from non-transformed WT (wt C90), empty plasmid-only transgenic control line (CK), and the 14 *B. carinata* T_3 transgenic lines expressing the *C. graeca KCS* gene (CG) under control of the napin promoter Values are presented as % wt/wt of each fatty acid ± SD. Data from Taylor *et al*.[110]

To this end, we have transformed *B. carinata* breeding line C90-1163 with a yeast *sn*-2 acyltransferase (*LPAT*) gene designated *SLC1-1* which had been shown to enhance oil content in *A. thaliana*, HEAR *B. napus* and *B. carinata*.[113] In field trials of the transgenic *B. carinata* harboring the yeast *LPAT* gene, oil content was increased about 3.5–5 percentage points, which translates to about a 12–16% relative oil content improvement, over the non-transformed control. This demonstrates the utility of using Kennedy pathway genes to enhance oil content in *B. carinata*. Recent work has shown that co-expressing the yeast acyltransferase with the *Crambe KCS* results in retention of increases in both oil content and erucic acid proportions (Table 7). Other anticipated transgene experiments will involve transformation with a set of *DGAT1* (*sn-3 acyltransferase*) genes from *Arabidopsis* and *T. majus*.[114]

Prospects and outlook

When considering potential platform crops for the delivery of bio-oils and industrial feedstocks, seed yield, oil and protein content are major considerations. Consequently, the plant must be more efficient in resource utilization while yield is maximized. *B. carinata* delivers high yields among the *Brassicaceae* (2500–3000 kg/ha). Despite this there are other concerns, the major targets for crop improvement being nutrient and water use efficiencies, resistance to biotic and abiotic stresses, increased biomass and carbon partitioning for increased harvest index. *B. carinata* is adapted to more adverse growing conditions compared to other oilseed *Brassicas*. However, it will only be a major contributor to the bioindustrial oil market if oil content can be maximized. The available knowledge of *Brassica* genomics should be used to increase understanding of basic plant biological processes and how these can be modulated for stress tolerance and increased oil yield. The exploitation of heterosis for increased yield and plant performance will not be possible without a better understanding of the process. Many agronomic traits affecting plant performance are complex and the use of genomic tools to enhance performance is a major undertaking. Useful genes for quantitative traits such

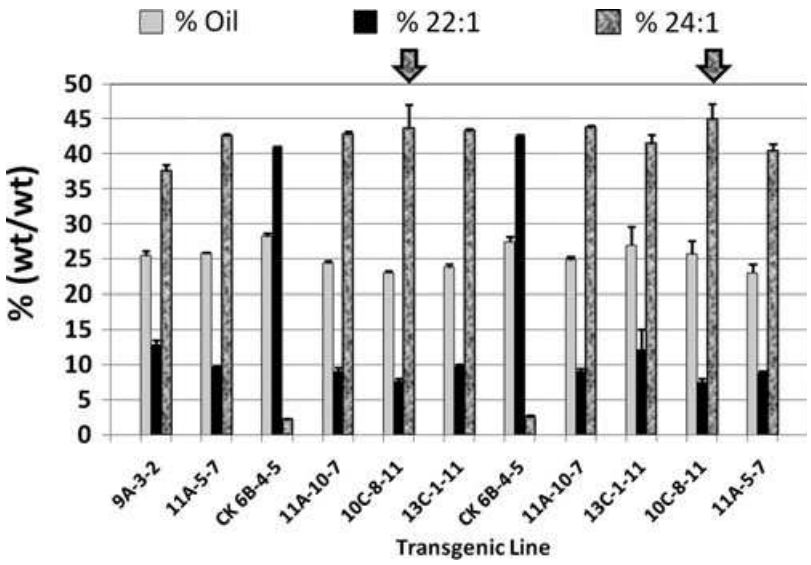

Figure 10. Confined field trial conducted in 2009 at Watrous, SK of transgenic *B. carinata* transformed with the *Cardamine graeca KCS* gene. Results from analyses of the T_4 seed samples from two replicates are shown for the empty plasmid-only transgenic control line (CK 6B-4-5) and the 4-5 best *B. carinata* T_3 transgenic lines expressing the *C. graeca KCS* gene (CG) under control of the napin promoter. 24:1 (% wt/wt; grey marbled bars), 22:1 (% wt/wt; black bars) and oil (% of DW; grey bars) are shown ±SD.

Table 6. Fatty acid composition of triacylglycerols (TAGs) of three selected T_2 seed lines of *B. carinata* expressing the *Lim Des5*. Data are expressed as % (wt/wt) total fatty acids ±SD. Tr, trace; <0.1%. Data from Jadhav *et al.*[112]

VLC Fatty Acid	% (wt/wt) of Total Fatty Acids in TAGs	% (wt/wt) of Total VLCFAs in TAGs
20:0	0.6 ± 0.01	1.1
20:1Δ5	1.0 ± 0.02	1.9
20:1Δ11	5.7 ± 0.1	10.6
20:2Δ5, Δ13	Tr	-
22:0	0.4 ± 0.01	0.7
22:1Δ13	**32.9 ± 0.1**	**60.9**
22:2Δ5, Δ13	**10.7 ± 0.02**	**19.8**
22:2Δ13, Δ16	1.2 ± 0.02	2.2
24:1Δ15	1.5 ± 0.01	2.8
Total C20, C22, C24	54.0 ± 0.29	100.0

Table 7. Relative seed oil content (as % of non-transformed control) and erucic acid content (% wt/wt), of T_2 generation of transgenic *B. carinata* breeding line C90-1163, transformed with the bakers' yeast *(Saccharomyces cerevisiae) SLC1-1 sn-2 acyltransferase (LPAT)* gene + the *Crambe KCS* gene. Shown are results from triplicate analyses of seed from the non-transformed control line (NT-Con) and T_3 seed from the five best *B. carinata* transgenic lines expressing the dual-gene construct under control of the napin promoter.

Line	Relative Oil Content (% of NT-Control)	22:1% (wt/wt)
NT-Con	100.0	41.1
3	110.6	49.5
15	115.4	44.9
16	117.2	48.8
23	134.8	43.5
33	125.3	46.7

as yield and oil content must be assembled in germplasm and used for variety improvement. Progenitors of *Brassica* species may be a valuable source of genes for improvement of *B. carinata*.[115,116]

It is interesting to note that in the examples cited above, we did not observe any significant changes in the

agronomic performance of the transgenics, compared to non-transformed *B. carinata* host germplasm – for example, % germination, date of emergence, time-to-flower, days-to-maturity and degree of lodging were all unaffected in the field. In our past experience, plant morphology would not be expected to be significantly altered in a transgenic plant wherein expression of the *KCS* gene is seed-specific, as with the napin promoter in the studies cited above. In contrast, in a previous experiment by Millar *et al.* [117] by over-expression of a different *Arabidopsis KCS* (*Fiddlehead* gene) in a constitutive manner (with a 35S promoter meaning it allowed expression in leaves and other vegetative tissues as well as developing seeds), the transgenic plants exhibited a dramatically altered morphology, which included the failure of flowering shoots to elongate (bolt), a modified spatial pattern of siliques, an altered flower phenotype and a unique alteration in the structure chloroplast membranes.

A key component of any new crop development is the question of whether it contributes positively to sustainable agriculture which has been defined as 'an integrated system of plant and animal production practices having site-specific application that will, over the long term, (1) satisfy human food and fiber needs, (2) enhance environmental quality and the natural resource base upon which the agricultural economy depends, (3) make the most efficient use of nonrenewable resources and on-farm resources and integrate, where appropriate, natural biological cycles and controls, (4) sustain the economic viability of farm operations, (5) enhance the quality of life for farmers and society as a whole'.[118]

A *B. carinata* crop platform can meet or exceed many of the targets for sustainable agriculture for the Prairies; 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, it is particularly noteworthy that the same *B. carinata* high nervonic oil can equally find direct applications 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 neuro-degenerative diseases.
- *B. carinata* is well suited to drier southern regions of the province/western Canada.
- Creation of a new crop platform adds genetic diversity, creates a new delivery system for bioindustrial and pharmaceutical oils that do not impact/compete with the food sector, specifically canola.
- *B. carinata* requires fewer inputs due to natural resistance to drought and blackleg; more robust architecture means stands are less prone to weediness.
- *B. carinata* provides the grower with enhanced yield (kg/ha) compared to 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 providing new opportunities as feedstocks for plastics and antigen delivery systems; the utility of both oil and meal are essential for complete utilization of the seed products, providing greater sustainability.

As indicated by the case studies 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 bioproducts. The application of genomic tools and biotechnological methods, notwithstanding the current state of uncertainty over the widespread acceptance of the latter, will allow introduction of a number traits for enhanced crop performance of *B. carinata*. As a platform crop, value can be enhanced by utilization of the entire crop, including the biomass, storage protein and minor seed components such as tocochromanols (e.g. vitamin E, a natural source of which may act as a better anti-oxidant compared to synthetic sources).[119] As an industrial crop, the potential for outcrossing of *B. carinata* with other *Brassica* species should be minimized. This will require knowledge of pollination biology to develop varieties with the appropriate pollen isolation systems. Besides the VLCFA examples discussed herein, we envision potential for delivery of other specialty molecules including very long-chain wax esters similar to those found in *jojoba*, and very long chain hydroxy fatty acids like those found in *Lesquerella*. Recent studies suggest that the oxidative stability of *B. carinata* oil makes it especially suitable for biodiesel.[13,120] It will be exciting to watch the evolution of *B. carinata* as a new industrial bioproducts platform crop in the years to come.

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