Development of *B. carinata* with super-high erucic acid content through interspecific hybridization

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Abstract

**Key message** Disomic alien chromosome addition *Brassica carinata* lines with super-high erucic acid content were developed through interspecific hybridization with *B. juncea* and characterized using molecular, cytological and biochemical techniques.

**Abstract** *Brassica carinata* [A.] Braun (BBCC, 2*n* = 34) is a climate-resilient oilseed. Its seed oil is high in erucic acid (> 40%), rendering it well suited for the production of biofuel and other bio-based applications. To enhance the competitiveness of *B. carinata* with high erucic *B. napus* (HEAR), lines with super-high erucic acid content were developed through interspecific hybridization. To this end, a *fad2B* null allele from *Brassica juncea* (AABB, 2*n* = 36) was introgressed into *B. carinata*, resulting in a *B. carinata fad2B* mutant with erucic acid levels of over 50%. Subsequently, the *FAE* allele from *B. rapa* spp. yellow sarson (AA, 2*n* = 20) was transferred to the *fad2B B. carinata* line, yielding lines with erucic acid contents of up to 57.9%. Molecular analysis using the *Brassica* 90 K Illumina Infinium™ SNP genotyping array identified these lines as disomic alien chromosome addition lines, with two extra A08 chromosomes containing the *BrFAE* gene. The alien chromosomes from *B. rapa* were clearly distinguished by molecular cytogenetics in one of the addition lines. Analysis of microspore-derived offspring and hybrids from crosses with a CMS *B. carinata* line showed that the transfer rate of the A08 chromosome into male gametes was over 98%, resulting in almost completely stable transmission of an A08 chromosome copy into the progeny. The increase in erucic acid levels was accompanied by changes in the proportions of other fatty acids depending on the genetic changes that were introduced in the interspecific hybrids, providing valuable insights into erucic acid metabolism in *Brassica*.

Introduction

*Brassica carinata*, also known as Ethiopian or Abyssinian mustard, is a member of the *Brassicaceae* (mustard) family. It is an allotetraploid (genome BBCC, 2*n* = 34) formed through the interspecific hybridization between the diploid progenitor species *B. nigra* (BB, 2*n* = 16) and *B. oleracea* (2*n* = 18) (U 1935; Prakash et al. 2011). Current evidence suggests that it originated in the highland plateaus of Ethiopia and neighboring areas of East Africa and the Mediterranean coast (Gomez-Campo and Prakash 1999; Alemayehu and Becker 2002). While the cultivation of Ethiopian mustard as an oilseed and vegetable crop in East Africa dates back several millennia (Simmonds 1979), interest in this crop in other geographic regions is relatively recent. This interest primarily stems from the well-documented heat and drought tolerance of this crop (Cohen and Knowles 1983; Ferreres et al. 1984; Malik 1990), and its potential to serve as a dedicated feedstock crop for biofuel production and other industrial applications (Taylor et al. 2010, Marillia et al. 2014).

*B. carinata* seed oil is high in erucic acid, with proportions ranging from 31 to 46% in natural germplasm collections (Röbelen and Thies 1980; Westphal and Marquard 1980; Becker et al. 1999; Warwick et al. 2006). Plant oils high in erucic acid are of interest for industrial purposes;
erucic acid and its derivatives are important renewable raw materials for the oleochemical industry, with more than 1000 potential or patented applications (Scarth and Tang 2006). Erucic acid (cis-13-docosenoic acid, C22:1) is a straight-chained mono-unsaturated very-long-chain fatty acid (VLCFA) with 22 carbon atoms and a double bond at the cis-13 position of the carbon chain. Biosynthesis of this fatty acid involves chain elongation of oleic acid (cis-9-octadecenoic acid, C18:1) to gondoic acid (cis-11-eicosenoic acid, C20:1) and then to C22:1 (Sanyal et al. 2015). This reaction is catalyzed by β-ketoacyl-CoA synthase (KCS), a membrane-bound enzyme that utilizes malonyl-CoA as the source of the two carbon atoms added in each cycle (Griffiths et al. 1998). KCS is encoded by the gene fatty acid elongase (FAE) (James et al. 1995; Lühs and Friedt 1997) and is the rate-limiting enzyme that plays a key role in determining the levels of erucic acid and other VLCFAs in seed oils (Millar and Kunst 1997). Early work in B. carinata proposed that C22:1 content is controlled by two loci in an additive manner with one allele present in each genome (Getinet et al. 1997). In agreement with this, it was demonstrated that two FAE genes determine the C22:1 content in the closely related species B. napus (Fourmann et al. 1998) and B. juncea (Venkateswari et al. 1999). Several studies, including experiments that involve interspecific hybrids, have demonstrated that the erucic acid content in Brassica seed oils is highly heritable (Getinet et al. 1997; Alemayehu and Becker 2001; Farhatullah et al. 2014).

Results from traditional breeding approaches in B. carinata suggest that C22:1 levels ranging from zero (Alonso et al. 1991; Getinet et al. 1994) to low (< 2%; Fernandez-Escobar et al. 1988; Velasco et al. 1995) to as high as 51% (Alemayehu and Becker 2001) are possible due to natural variation and through selection breeding, compared to <1 to 60% in non-canola types of B. rapa and B. napus (Lühs and Friedt 1994; Lühs et al. 1999) and <1 to 52% in B. juncea (Saikia et al. 2018). Several studies in various Brassica species have manipulated C22:1 levels via mutagenesis or transgenic approaches. Thus, chemical mutagenesis has been used to both reduce and elevate C22:1 levels in B. carinata (Barro et al. 2001). UV treatment has also been used to increase C22:1 levels from 42.8 to 49.5% (Barro et al. 2003). Both sense and antisense FAE constructs were used in B. napus (Zebzarjadi et al. 2006) and B. juncea (Kanrar et al. 2006) to increase and decrease the C22:1 content, respectively. In B. napus cultivar CY2, an RNAi approach reduced the C22:1 content from 40 to 3%, with corresponding increases in C18:1 from 20 to 60% (Shi et al. 2015). C22:1 levels were increased in B. carinata by either a co-suppression or antisense construct of omega-6 fatty acid desaturase (FAD2) (Jadhav et al. 2005) through an increase in the amount of the substrate C18:1. Further, silencing of FAD2 via a hairpin construct in combination with a Crambe abyssinica FAE construct in B. carinata resulted in levels of C22:1 as high as 56% (Mietkiewska et al. 2008).

While genetic transformation is a powerful means of effectively transferring genes across reproductive barriers (Cardi et al. 2017), genetically modified crops are facing regulatory and trade challenges in contrats to crops developed through traditional breeding (Smyth 2016). Serendipitously, in the Brassica genus, several crop species have genomes in common and the ability to perform interspecific hybridizations allows for the transfer of useful adaptive traits between species for targeted crop improvement through conventional breeding (Katche et al. 2019). Interspecific crosses with the compatible allotetraploid species B. napus and B. juncea have resulted in B. carinata lines with decreased glucosinolate content (Márquez-Lema et al. 2008) and increased seed oil content (Sheikh et al. 2009), as well as increased overall genetic diversity (Sheikh et al. 2011). Similarly, C22:1 levels in B. carinata were significantly reduced through crosses to both B. napus (Fernandez-Escobar et al. 1988) and B. juncea (Getinet et al. 1994). On the other hand, B. carinata has been used as a donor for the transfer of a number of traits, such as blackleg (Leptosphaeria maculans) resistance to B. napus (Fredua-Agyeman et al. 2014; Navabi et al. 2010) and resistance to white rust (Albugo candida) and Alternaria blight (Alternaria brassicaca) to B. juncea (Gupta et al. 2010). Several groups have utilized B. carinata to introduce yellow seed coat color into B. napus (Rashid et al. 1994; Meng et al. 1998; Rahman 2001). In addition, overall genetic diversity has been enhanced in both B. napus (Chen et al. 2010a, b) and B. juncea (Wei et al. 2016) through crosses with B. carinata. Interspecific crosses of B. carinata with diploid Brassica species have also been explored. The transfer of powdery mildew (Erysiphe polygoni) (Tonguç and Griffiths 2004) and black rot (Xanthomonas campestris) resistance (Sharma et al. 2017) from B. carinata to B. oleracea was examined in early backcross generations; both studies required embryo rescue. Crosses with B. rapa were found to be challenging and highly dependent on the accessions used (Jiang et al. 2007) and the direction of the cross (Choudhary et al. 2000), although successful crosses between B. rapa and B. carinata have been achieved in both directions (FitzJohn et al. 2007; Tian et al. 2010). In particular, successful crosses with diverse B. rapa genotypes including brown sarson, toria and yellow sarson have been achieved (Choudhary et al. 2000; Rahman 2001); yellow sarson is known to have erucic acid levels greater than 55% (Dorrell and Downey 1964).

Industrial applications demand an oil composition predominated by a single fatty acid. Compared to low-erucic Brassica oils, high erucic oils have a higher energy potential (Semenov et al. 2006) and are more desirable substrates for biodiesel (Vicente et al. 2005) and biofuel (Zhao et al. 2016) production. High erucic acid rapeseed (HEAR), currently
the main feedstock for erucic acid used in industrial applications, contains about 50% of this fatty acid (Sanyal et al. 2015). For improved technical utility and enhanced marketability of carinata oil, it is desirable to increase the C22:1 content and to simultaneously decrease the content of C20:1, polyunsaturated fatty acids (PUFA, linoleic [C18:2] and linolenic [C18:3] acids) and saturated fatty acids (palmitic [C16:0] and stearic [C18:0] acids).

The purpose of the present study was to create a B. carinata breeding line with super-high C22:1 content through interspecific hybridization with B. juncea and B. rapa. Herein, we report the development of a B. carinata disomic A genome chromosome addition line, carrying the fad2B/FAE gene variants, with erucic acid levels of close to 60%, and its characterization using molecular, cytological and biochemical techniques. Further, we demonstrate the almost completely stable transfer of the chromosome addition through crosses with cytoplasmic male sterile B. carinata and the production of offspring via isolated microspore culture. This study provides the first account of the development of super-high erucic acid Ethiopian mustard through conventional breeding and contributes to the understanding of erucic acid metabolism in Brassica. It is also the first report to describe a B. carinata-rapa disomic chromosome addition line.

Materials and methods

Plant materials and breeding strategy

In previous work, the high erucic acid B. carinata breeding line 080793EM was crossed with canola-quality B. juncea line VR07-358 to increase the C18:1 content by introducing a fad2B null allele (Roslinsky et al. 2011). In the present study, the BC2F3 progeny line VR10-183.8 was crossed to the yellow sarson B. rapa line R500 in order to introduce the B. rapa FAE gene (BrFAE) (Fig. 1). The resulting F1 plants were backcrossed to VR10-183.8 and embryos rescued 16 days after pollination (DAP) according to the method described below. A second backcross was completed, followed by two rounds of self-pollination. The BC2F3 line VR13-156 was identified as a stable double mutant for the fad2B null allele and the B. rapa-derived FAE gene via genotyping analysis, and was subsequently crossed to the doubled haploid (DH) B. carinata breeding line 111078EM-7 to improve total seed oil content and overall agronomics. Improvements to the FAD2-specific marker allowed for F1 lines to be used in the ensuing crossing procedure. Three backcrosses were carried out with 111078EM-7 as the recurrent parent, followed by three rounds of self-pollination. To determine the transfer rate of the B. rapa FAE gene during meiosis, BC3F2 line VR16-072 was used as the donor line for the production of microspore-derived plants. Additionally, individual BC3F4 lines were crossed to a cytoplasmic male sterile (CMS) line to examine pollen viability as well as segregation of the BrFAE allele in the F1 progeny. BC3F4 line VR17-095 was used for the molecular cytogenetic study. BC3F4 lines VR17-089 and VR17-095 were analyzed on a Brassica 90K Illumina Infinium™ SNP genotyping array. Parent material and progeny lines were grown in 2019 in the field in a randomized complete block design with four replicates alongside the two commercial B. carinata cultivars AAC A110 and AAC A120 (Agriculture and Agri-Food Canada—Saskatoon Research and Development Centre, Canada). Harvested seed was subjected to fatty acid analysis.

Embryo rescue

F1 hybrids resulting from the cross between VR10-183.8 and yellow sarson B. rapa line R500 were used as the female in backcrosses with the recurrent parent VR10-183.8. BC1F1 plants were developed by embryo rescue followed by in vitro culture according to the protocol of Ayotte et al. (1987). Plantlets were transferred to soil and placed in a greenhouse.

FAE and FAD2 gene sequencing and marker development

Genomic DNA was isolated from young leaf tissue of the parents and progeny lines using a sodium dodecyl sulfate protocol as per Somers et al. (1998). The primer pair FAE F and FAE R (Online Resource 1) was designed to amplify all FAE alleles present in a sample, based on Gupta et al. (2004). All PCR reactions were carried out as follows: genomic DNA was used as the template for PCR amplification in a thermocycler with 35 cycles of 45 s for denaturation at 94 °C, primer annealing at 57 °C and 1 minute extension at 72 °C. The PCR products from the parental lines R500 and VR10-183.8 were subsequently cloned and sequenced; the expected bands were cloned into the pGEM-T Vector System I (Promega, Cat. No. A3600) and plasmids were extracted using the QIAprep Spin Miniprep Kit (Qiagen, Cat. No. 27106) following the manufacturer’s recommended protocols. All samples were sequenced at the National Research Council in Saskatoon, Canada. Screening of early generations for the presence of BrFAE was carried out with an HpaI post-amplification digest using the above primer pair. The digest was run on a 2% agarose gel for 3 hours at 120 v to ensure adequate separation of fragments. To improve screening capacity, KASP primers (LGC Genomics) were developed for routine screening utilizing the HpaI SNP position (Fig. 2) and implemented at the first backcross to recurrent parent 111078EM-7 and subsequent generations. The KASP reaction contains 100 ng genomic DNA,
Fig. 1 Schematic representation of the breeding strategy for the development of the super-high erucic acid *B. carinata* lines VR13-156 and VR17-095.

Note: Line VR10-183.8 previously developed by (Roslinsky et al. 2011)
4.0 μL KASP 2x Master Mix (LGC Genomics, Cat. No. KBS-1016-002-US) and 0.11 μL primer assay mix (Online Resource 1) in a total volume of 8 μL. All amplifications were performed in a CFX96 Real-Time Thermal Cycler (Bio-Rad Laboratories) using a touchdown PCR protocol as per the manufacturer’s recommended procedure.

Primer pair fad2B F and fad2B R (Online Resource 1) was designed to amplify the FAD2B allele. The amplification followed the same PCR protocol as described above, except that the annealing temperature was 64 °C. With this primer pair, selection against the amplified product was required to select the fad2B null allele. As more sequence information was obtained later in the pedigree, new primers were developed facilitating a co-dominant marker system. This new primer pair (fad2B F2 and fad2B R2) (Online Resource 1) amplified a region tightly linked to FAD2 present in all samples. The PCR protocol is again similar to the one described above, with an annealing temperature of 55 °C. Amplified products for both primer pairs were separated on 2% agarose gels and visualized through ethidium bromide staining. All samples at the BC2F1 × 111078EM-7 stage and beyond were tested with the second FAD2 primer set.

**Seed fatty acid analysis**

Seed fatty acid composition of field-grown parental and progeny lines was analyzed according to Thies (1971) with the following modifications: methyl esters were separated by gas chromatography using an Agilent INNOWAX fused silica capillary column (7.5 m x 250 μm diameter x 0.5 μm film thickness) at 250 °C with hydrogen as the carrier gas. The methyl esters were prepared by base-catalyzed transesterification using sodium methoxide in methanol and hexane on oil extracted from 3.0 g of crushed seed.

**Genotyping**

Parental and progeny lines were analyzed using the Brassica 90K Illumina Infinium™ SNP genotyping array as per the supplied protocol. Data analysis was performed utilizing the GenomeStudio software package v 2.0 (https://support.illumina.com/array/array_software/genomestudio/documentation.html) according to suggested best practices (Mason et al. 2017; Clarke et al. 2016).

B. carinata B

\[\text{AAAAATCTATTCGAGACACCAAGGTTAAGCCTAAAGATATTGATATCTTGTTGGAACCTCA}\]

B. carinata C

\[\text{AAAAATCTATTCGAGACACCAGGTTAAGCCTACGATATTGATATCTTGTGTTGGAACCTCA}\]

B. rapa

\[\text{AAAAATCTATTCGAGACACCAACGTTAAGCCCTACTATTTGATATCTTGTTGGAACCTCA}\]

To determine the transfer rate of BrFAE during meiosis, BC2F1 line VR16-072, derived from cross VR13-156 × 111078EM-07, was used as the donor line for the production of microspore-derived plants. Isolated microspore culture was performed as described in Zatynly et al. (in press). Germinated embryos with true leaves and roots were transplanted to pots and transferred to the greenhouse.

**Molecular cytogenetics**

Root tips were collected and chromosome spreads prepared from root tips following the protocol described in Gaebelein et al. (2019), adapted from Snowdon et al. (1997). Cells were observed using a Leica DRME microscope equipped with a Leica EL6000 fluorescent light source. Pictures were taken using a Leica DFC450 C camera and Leica Application Suite X software. Grayscale images showing the red and green fluorescent signals were processed to achieve optimal signal strength and contrast in ImageJ and combined. The combined image was then overlaid with the original grayscale image of the respective cell taken before hybridization using Photoshop CS6.

Labeled probe to detect B genome chromosomes was prepared using Roche Dig-Nick Translation Mix (Cat. No. 11745 816 910) according to the manufacturer’s specifications, using genomic B. nigra DNA as a template. Labeled probe to detect C genome chromosomes was prepared using the Invitrogen BioPrime DNA Labelling System (Cat. No. 18094011) according to the manufacturer’s specifications using DNA from BAC (Bacterial Artificial Chromosome) clone BoB014006 (Howell et al. 2008) as template DNA, which contains the C genome-specific repetitive element Bot1 (Alix et al. 2008), which is distributed across all C genome chromosomes. Fluorescent in situ hybridization was carried out according to the methods detailed in Gaebelein et al. (2019), as adapted from Leffon et al. (2016). 100 ng of B genome DNA and 200 ng of C genome-specific BAC DNA were used in the hybridization mix, and 2 ng/μl Anti-Digoxigenin-FITC (Roche, Cat. No. 11207741910) and 10 ng/μl Cy3-Streptavidin (GE-Healthcare, Cat. No. PA43001) dissolved in 5% BSA (Bovine Serum Albumin) were applied for visualization of the antibody-labeled DNA.

**Fig. 2** KASP primer positions in the FAE gene sequence. The targeted SNP at position 506 in the fragment is highlighted in blue, the sequence targeted by the selective primer in yellow and the sequence targeted by the common primer in green. The bases in red represent variation in the B allele, while bold and underlined bases were utilized for further selective amplification of the BrFAE sequence.
probes. DAPI (4′,6-diamidino-2-phenylindole)-containing medium (Vectashield H-1200) was used to visualize the chromosomes under UV-light excitation.

**Statistical analysis**

Fatty acid profile data were analyzed by ANOVA using the Glimmix Procedure of SAS (SAS for Windows 9.3, SAS Institute, USA). Treatment mean comparisons were made using the Tukey–Kramer method at the $P = 0.05$ level.

**Results**

**Development and characterization of super-high erucic acid B. carinata**

In *B. carinata* BC3F3 breeding line VR10-183.8 a B1/A5 translocation event had removed a portion of chromosome B1 that contained the *FAD2B* allele (Roslnsky et al. 2011), as a result of the cross with canola-quality *B. juncea* (Potts et al. 1999). This translocation caused an increase in the C18:1 content, combined with an increase in C22:1, and reductions in both C18:2 and C18:3 levels. In the present study, crosses were initiated to combine the *fad2B* null allele with the *FAE* gene from the yellow sarson *B. rapa* line R500. The BC1 crosses were embryo rescued to obtain viable interspecific hybrids. All following generations naturally set varying amounts of seed. At the BC2F3 stage, the breeding line VR13-156 was identified to be a double mutant homozygous for both the *fad2B* null allele and the rapa-derived *FAE* allele. This line showed a further significant increase in C22:1 content compared to the parental line VR10-183.8. At the same time, this line displayed poor overall growth and seed set, and had a low total seed oil content. Three additional rounds of backcrossing to the elite *B. carinata* breeding line 111078EM-7 and three cycles of self-pollination were completed to develop true-breeding double mutant lines with improvements in agronomic and seed quality traits. BC3F4 line VR17-095 was selected based on superior seed yield in the greenhouse (unpublished data).

**FAE and FAD2 sequence analysis and marker development**

A 1254 bp portion of the *FAE* genes from the breeding lines VR10-183.8 and R500 was amplified and sequenced using primers based on data from Gupta et al. (2004). A SNP was detected in an *HpaI* digestion site at position 506 of the fragment which is present in both *B. carinata* endogenous *FAE* alleles, but absent in the R500 allele. Originally, this SNP was utilized in a post-amplification restriction digest that produced a unique 538 bp band specific for the *BrFAE* allele (Online Resource 2). To improve marker efficiency, this SNP was converted to a KASP marker. As three homoeologous copies of the *FAE* gene are present (*BcFAE-B, BcFAE-C* and *BrFAE*), both the selective and common primers were designed to preferentially amplify *BrFAE* to ensure adequate separation of the allele clusters and to capture all rare A genome insertion events (Fig. 2). This marker design is not typical of KASP markers and does not allow for the identification of heterozygous samples (Fig. 3).

The *fad2B* null allele was identified in canola-quality *B. juncea* and transferred to *B. carinata* (Roslnsky et al. 2011). The molecular marker (Genbank DQ777854) that was initially developed targets the *FAD2B* gene itself and amplifies a single 456 bp fragment (Online Resource 3). Because the desired genotype is the homozygous null allele and selection for the absence of amplification was required, selections could only be made in an F2 generation. This resulted in one additional round of selfing after each backcross. In order to save time and avoid the occurrence of false negatives, a second marker was designed based on the sequence of a non-coding fragment associated with the B1/A5 translocation (Figure S2). When analyzed with this marker, the *B. carinata* lines developed in this study contain a 473 bp fragment associated with the wild-type *FAD2B* allele, a 615 bp band associated with the A5 translocation and the *fad2B* null allele and a 440 bp fragment that appears to be associated with the *FAD2C* allele (Fig. 4). Because of the co-dominant nature of this marker, the selection of lines homozygous for the *fad2B* null allele is readily feasible.
The FAE and FAD2 markers described herein were used throughout the pedigree to identify stable double mutants homozygous for the juncea-derived fad2B null allele and the rapa-derived FAE gene.

**Seed fatty acid analysis**

The lines representing important steps in the pedigree alongside samples of two commercial B. carinata cultivars, AAC A110 and AAC A120, were grown in replicate in a field trial at Saskatoon, Saskatchewan, and their seed fatty acid profiles were analyzed by gas chromatography. The relative amount (percentage of total fatty acids) of the major fatty acids C18:1, C18:2, C18:3, C20:1, C22:1 and nervonic acid (C24:1) as well as the percentage of unsaturated fatty acids is presented in Table 1. The fatty acid profiles of cultivars AAC A110 and AAC A120 are representative of generic B. carinata germplasm, while the seed oil of breeding line 080793EM is characterized by elevated C22:1 levels (net increase of 4.9% and 3.9% compared to AAC A110 and AAC A120, respectively) and lower amounts of C18:1, C18:3 and C24:1. The results of the analysis show that fad2B line VR10-183.8 possesses a C18:1 content that is 3.3% higher than that in B. carinata parent line 080793EM. This increase in C18:1 is accompanied by a 4.3% increase in C22:1 and reductions in both C18:2 and C18:3. Both C20:1 and C24:1 levels remain unchanged. BC1F3 line VR13-156, the first line in the pedigree identified as homozygous for both the fad2B null allele and BrFAE, shows a further increase in C22:1 levels with values that are on average 11% above those in line 080793EM (57.9 vs. 46.9%) which corresponds to a proportional increase of 23%. Compared to VR10-183.8, line VR13-156 is characterized by an increased amount of C22:1 (57.9 vs. 51.2%) and proportional reductions of C18:3 and C20:1 levels, by 20.8 and 59.7%, respectively. Line VR13-432, a direct progeny line of VR13-156 derived through selfing, features a fatty acid profile very similar to that of its parent with a statistically significant difference, albeit small, only in C18:1 levels. Line 111078EM-7, used as the recurrent parent in backcrosses to VR13-156, has a fatty acid profile that is very similar to that of the two commercial cultivars included in this study. Interestingly, BC3F4 lines VR17-095 and VR17-89 have C22:1 levels that are 9.8% and 9.4% above those of the recurrent parent and 3.5 and 3.9% below those of donor parent VR13-156. In all fad2B/FAE lines the percentage of saturated fatty acids was slightly but significantly reduced when compared to that of the parent lines 080793EM and 111078EM-7, respectively.

**Genotyping**

In order to determine the exact position and size of the BrFAE introgression, the BC3F4 lines VR17-089 and VR17-095 were analyzed using the *Brassica* 90K Illumina Infinium™ SNP genotyping array. FAE has previously been mapped to the A08 chromosome in B. napus (Fourmann et al. 1998; Delourme et al. 2006) and it was therefore expected that a portion of the A08 chromosome would be introgressed into the genome of B. carinata. The array data revealed that the entire B. rapa A08 chromosome was present, rendering VR17-089 and VR17-095 disomic alien chromosome addition lines (BBCC+A08). The array data...
also showed that the distal portion of chromosome B1 was replaced with a distal portion of chromosome A5 (Figure S2). This re-arrangement led to the loss of the \( \text{FAD2B} \) allele, making VR17-089 and VR17-095 \( \text{fad2B/FAE} \) \( \text{B. carinata} \) disomic A-chromosome addition lines.

**Molecular cytogenetics**

Unambiguous counts of 36 chromosomes were found in 50 cells from 23 independent plants of line VR17-095, supporting a stable karyotype of \( 2n = 36 \) in this line. Three microscope slides were successfully hybridized with probes specific to the \textit{Brassica} B and C genomes, with sufficient signal strength, probe specificity and limited degradation of chromosomes on the slides. Fifteen chromosome spreads with good chromosome spreading and chromosome condensation were found to show 16 clear signals representing the B genome chromosomes, 18 clear signals representing the C genome chromosomes and two distinctly unstained chromosomes putatively representing the chromosome pair A08 introgressed from \textit{B. rapa} (Fig. 5).

| Line                  | Fatty acids (%) of total | Total Sat. | C18:1 | C18:2 | C18:3 | C20:1 | C22:1 | C24:1 |
|-----------------------|--------------------------|------------|-------|-------|-------|-------|-------|-------|
| AAC A110\(\text{**f**}\) | 5.4 c 7.4 d 15.3 a 15.7 a 7.1 ab 42.0 f 2.9 ab |            |       |       |       |       |       |       |
| AAC A120\(\text{**f**}\) | 5.7 b 7.4 d 14.7 ab 14.6 b 7.7 a 43.0 ef 2.8 b |            |       |       |       |       |       |       |
| 080793EM              | 5.9 a 6.3 f 15.4 a 13.0 c 6.2 b 46.9 d 1.9 c |            |       |       |       |       |       |       |
| VR10-183.8            | 5.2 c 9.6 a 10.8 e 12.0 d 6.7 b 51.2 c 1.8 cd |            |       |       |       |       |       |       |
| VR13-156\(\text{**f**}\) | 4.9 d 9.0 b 11.7 cd 9.5 e 2.7 d 57.9 a 1.6 e |            |       |       |       |       |       |       |
| VR13-432              | 4.8 d 8.5 c 12.0 c 9.9 e 2.8 cd 57.4 a 1.7 de |            |       |       |       |       |       |       |
| 111078EM-7            | 5.3 c 6.7 ef 14.2 b 15.6 a 6.2 b 44.6 e 3.0 a |            |       |       |       |       |       |       |
| VR17-095              | 4.8 d 6.8 ef 11.4 d 12.9 c 3.4 cd 54.4 b 2.7 b |            |       |       |       |       |       |       |
| VR17-089              | 4.8 d 6.9 de 11.9 cd 12.5 cd 3.5 c 54.0 b 2.7 b |            |       |       |       |       |       |       |

Means within a column with different lowercase letters differ significantly at the \( p < 0.05 \) level

AAC A110, AAC A 120, 080793EM and 111078EM-7: generic \textit{B. carinata} cultivars and breeding lines, respectively; VR10-183.8: \textit{fad2B} null line derived from \textit{B. carinata} 080793EM x \textit{B. juncea}; VR13-156: double mutant line homozygous for \textit{fad2B} and \textit{B. rapa} \textit{FAE}, \( \text{BC}_2F_1 \) derived from VR10-183.8 x R500; VR13-432: double mutant line homozygous for \textit{fad2B} and \textit{B. rapa} \textit{FAE}, \( \text{BC}_3F_4 \) derived from selfing of VR13-156; VR17-089 and VR17-095: double mutant line homozygous for \textit{fad2B} and \textit{B. rapa} \textit{FAE}, \( \text{BC}_3F_4 \) lines derived from VR13-156 x 111078EM-7

**Stability of the \textit{B. rapa}-derived \textit{FAE} introgression**

To determine the transfer rate of the \textit{BrFAE} gene during meiosis, \( \text{BC}_3F_2 \) line VR16-072 was used as a donor line for the production of microspore-derived plants. Of the 171 plantlets that were tested, 168 contained the \textit{BrFAE} allele, which corresponds to a transfer rate of 98.25%. Further, in order to confirm that the pollen of the chromosome addition lines is viable and not selected against during pollination, crosses were carried out between 12 \( \text{BC}_3F_4 \) plants and a CMS \textit{B. carinata} line. Seed set after pollination was normal. To confirm the transfer of the \textit{BrFAE} allele, eight \( \text{F1} \) plants from each of the 12 cross combinations were tested for its presence using the above described markers, with 95 of the 96 lines containing the \textit{BrFAE} gene, equating to a transfer rate of 98.96%.

**Discussion**

Erucic acid is one of several hundred unusual fatty acids produced by plants (Ohlrogge et al. 2018) and is found almost exclusively in triacylglycerols (TAGs) in the seed oil of the \textit{Brassicaceae} family and the genus \textit{Limnanthes} (meadowfoams) (Shi et al. 2015). C22:1 and its derivatives

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Table 1 Mean values for fatty acids as percentage of total fatty acids of the seed oil of \textit{Brassica carinata} parent and progeny lines grown in a field trial at Saskatoon, Saskatchewan, in 2019; \( n = 4 \) unless specified otherwise.
are important industrial feedstocks. Applications include the use of erucamide as a slip-promoting agent in plastic manufacturing and the production of high temperature lubricants, detergents, emulsifiers, photographic materials, as well as cosmetics and even pharmaceuticals (Leonard 1994; Sonntag 1995; Piazza and Foglia 2001; McVetty and Scarth 2002; Impallomeni et al. 2011). According to Li et al. (2012), it is estimated that a 10% increase in C22:1 content of total seed fatty acids results in a 50% reduction of the costs of erucamide production. The plethora of applications and the economics of production in combination with the need for improved technical utility and enhanced marketability of carinata oil have motivated our efforts in the development of transgenic soybean and cotton as well as canola-quality B. napus and B. juncea lines (Stoutjesdijk et al. 2000; Kinney et al. 2002; Liu et al. 2002). It appears that the increased pool of C18:1 in VR10-183.8 was utilized by the endogenous fatty acid elongases (BcFAE-B, BcFAE-C), resulting in a simultaneous increase of the proportion of C22:1 to over 50% of total fatty acids. Thus, the reductive effect of the fad2B null allele on C18:1 desaturation may have enhanced substrate availability for the biosynthesis of C22:1 via elongation. This finding is in agreement with results from earlier work in Arabidopsis thaliana (Okuley 1994), B. rapa (Bao et al. 1998) and B. napus (Domergue et al. 1999). Jadhav et al. (2005) downregulated the expression of FAD2 in B. carinata using co-suppression and antisense silencing approaches. While the resulting transgenic carinata lines had significantly increased C22:1 levels, none reached levels above 46% of total fatty acids. In addition, the effectiveness of these particular transgenic strategies is variable and unpredictable and they require the production of large transgenic populations to obtain an adequate number of lines showing sufficient levels of target gene suppression (Knutzon et al. 1992; Kinney 1996; Liu et al. 2002). Interestingly, in contrast to our results and the above cited studies, down-regulation of FAD2 in Crambe abyssinica by RNAi did not increase the C22:1 content, suggesting that oleate availability is not a rate-limiting factor in the biosynthesis of this fatty acid in crambe (Li et al. 2012; Cheng et al. 2013).

Line VR13-156, which in addition to the fad2B null allele possesses a heterologously expressed B. rapa FAE gene, exhibited an additional increase in C22:1 levels to just under 58%. Millar and Kunst (1997) were the first to show that introducing A. thaliana FAE1 in A. thaliana or Nicotiana tabacum (tobacco) tissue void of significant quantities of VLCFAs resulted in their accumulation and that increasing gene dosage through introduction of extra copies of FAE1 resulted in higher levels of these fatty acids. Since then, various FAE genes encoding enzymes with different acyl-CoA specificity have been identified, cloned and overexpressed in plants to increase C22:1 levels in the seed oil. Thus, overexpression of a FAE gene from Tropaeolum majus (garden nasturtium) in A. thaliana led to an increase in C22:1 proportions in the seed oil of up to eight-fold (Mietkiewska et al. 2004). In the present study, the very high proportion of C22:1 in the seed oil of fad2B/FAE line VR13-156 was associated with lower proportions of C18:3 and C20:1 compared to seed oil of VR10-183.8. This indicates that the new heterologous BrFAE acts in concert with the endogenous B. carinata FAE enzymes in elongating C18:1 to C22:1, which would limit the pool of C18:1 available for desaturation, as suggested by Mietkiewska et al. (2007).

Similarly, suppression of FAD2 expression has been used successfully to increase the proportions of C18:1 in transgenic soybean and cotton as well as canola-quality B. napus and B. juncea lines (Stoutjesdijk et al. 2000; Kinney et al. 2002; Liu et al. 2002). It appears that the increased pool of C18:1 in VR10-183.8 was utilized by the endogenous fatty acid elongases (BcFAE-B, BcFAE-C), resulting in a simultaneous increase of the proportion of C22:1 to over 50% of total fatty acids. Thus, the reductive effect of the fad2B null allele on C18:1 desaturation may have enhanced substrate availability for the biosynthesis of C22:1 via elongation. This finding is in agreement with results from earlier work in Arabidopsis thaliana (Okuley 1994), B. rapa (Bao et al. 1998) and B. napus (Domergue et al. 1999). Jadhav et al. (2005) downregulated the expression of FAD2 in B. carinata using co-suppression and antisense silencing approaches. While the resulting transgenic carinata lines had significantly increased C22:1 levels, none reached levels above 46% of total fatty acids. In addition, the effectiveness of these particular transgenic strategies is variable and unpredictable and they require the production of large transgenic populations to obtain an adequate number of lines showing sufficient levels of target gene suppression (Knutzon et al. 1992; Kinney 1996; Liu et al. 2002). Interestingly, in contrast to our results and the above cited studies, down-regulation of FAD2 in Crambe abyssinica by RNAi did not increase the C22:1 content, suggesting that oleate availability is not a rate-limiting factor in the biosynthesis of this fatty acid in crambe (Li et al. 2012; Cheng et al. 2013).

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Fig. 5 FISH/GISH stained mitotic cell of VR17-095 with a karyotype of 2n = 36. Green (fluorescein) signals represent chromosomes stained by probe derived from genomic DNA of B. nigra, identifying B genome chromosomes. Red signals (Cy3) represent chromosomes stained by probe derived from repetitive DNA sequence of B. oleracea, identifying C genome chromosomes. Two distinct small chromosomes (arrows) remain unstained, identifying them as putative A genome chromosomes.
FAE enzymes in elongating C18:1 to C22:1, a hypothesis corroborated by the naturally very high C22:1 proportions in seed oil of yellow saxon B. rapa line R500 (Dorrell and Downey 1964), the source of BrFAE in this study. Further, it is tempting to speculate that the BrFAE enzyme shows stronger elongase activity with gondoyl-CoA than with oleoyl-CoA, explaining the decrease in C20:1 levels in VR13-156 compared to those in VR10-183.8. The co-expression of elongases with different but complementary substrate preferences has been used successfully in a transgenic approach to increase the production of C22:1 in seeds of B. carinata (Mietkiewska et al. 2004), albeit not to the levels reported in this study.

While the introgression of the fad2B null allele alone results in a significant increase in the C18:1 content in VR10-183.8 compared to parent line 080793EM, the simultaneous introduction of this gene and BrFAE into wild-type 111078EM-7 did not yield significant changes in the content of this fatty acid in the lines VR17-089 and VR17-095. This may be explained by the immediate elongation of the increased pool of C18:1 through the heterologous BrFAE in seeds of the double mutant lines. This proposition is substantiated by the observation of Mietkiewska et al. (2007) that the C18:1 elongation pathway has a somewhat stronger ‘metabolic pull’ over C18:1 desaturation in developing seeds.

To the best of our knowledge, the current introgression of the B. juncea fad2B null allele and FAE gene from B. rapa has resulted in the highest level of C22:1 documented thus far for B. carinata seed oil. In comparison, the best result obtained through transgenic manipulations of B. carinata was observed in an RNAi FAD2/Crambe FAE transgenic line with 56% C22:1 (Mietkiewska et al. 2008). The C22:1 levels in double mutant lines VR17-089 and VR17-095 are lower than those in VR13-156 very likely because the recurrent parent 111078EM-7 they originated from also has a lower C22:1 content than line 080793EM which was used in the first cross with B. juncea. This stresses the importance of giving consideration to the level of the target fatty acid in the genetic background to be improved, in this case the recurrent parent, when attempting to develop germplasm with substantially increased proportions of that fatty acid.

Theoretically, the highest level of erucic acid that can be achieved in B. carinata seed oil through conventional breeding is 66.7%. This is because as in most Brassicaceae, in B. carinata the endogenous lysophosphatidic acid acyltransferase (LPAT), the enzyme responsible for placing acyl moieties in the sn-2 or middle position on the glycerol backbone during triacylglycerol biosynthesis via the Kennedy pathway (Kennedy 1961), cannot use erucic acid as a substrate (Frentzen 1993; Kuo and Gardner 2002; McVetty and Scarth 2002). In order to further increase the erucic acid content in our fad2B/FAE carinata lines, one option might be to introduce a diacylglycerol acyltransferase (DGAT) from a closely related Brassica species. DGATs catalyze the final step of triacylglycerol synthesis and it has been shown that specialized DGAT2s play a key role in channeling unusual acyl groups into TAGs (Shockey et al. 2006; Burgal et al. 2008; Li et al. 2010). In this scenario, the addition of an extra Brassica DGAT2 gene with high activity toward erucic acid would allow the plant to more efficiently channel erucic acid into TAG, as discussed by Demski et al. (2019).

In the present study, the introgression of BrFAE into the B. carinata genome was achieved through the transfer of the entire B. rapa A08 chromosome during the process of backcrossing. Almost completely stable fad2B/FAE B. carinata alien chromosome addition lines carrying two copies of chromosome A08 were produced and maintained. Alien chromosome addition lines in general are characterized by the addition of one (monosomic) or two (disomic) copies of a certain chromosome from one species to the genome of another species (Budahn et al. 2006). In Brassica, alien chromosome addition lines have been used extensively to assign linkage groups to chromosomes, study genomic relationships within the genus and to transfer agronomically important traits between species (Budahn et al. 2006; Chen et al. 1997; Fan and Tai 1985; McGrath and Quiros 1990; Quiros et al. 1987; Struss et al. 1991), with numerous studies particularly on the development and characterization of B. napus-nigra monosomic chromosome addition lines (AACC + 1B) (Chevre et al. 1997; Jahier et al. 1989; McGrath and Quiros 1990; Struss et al. 1991, 1992; Zhu et al. 1993). Successful examples of gene introgression from more distantly related species through monosomic addition lines include the transfer of resistance to Leptosphaeria maculans from Sinapis arvensis (wild mustard) (Snowdon et al. 2000) and resistance to the beet cyst nematode from Raphanus sativus (oil radish) (Peterka et al. 2004) into B. napus. While monosomic addition lines have proven to be useful in the dissection of genomes and studying chromosomal homoeologies, their practical utility in plant breeding programs is limited by segregation of the alien chromosome in offspring and potential loss of chromosome integrity by homoeologous recombination (Budahn et al. 2006). Thus, within the Brassica genus, transmission rates of alien monosomic chromosomes vary greatly and are usually below 50% (Budahn et al. 2006; Chen et al. 2010a, b; Chevre et al. 1997).

In contrast, disomic addition lines, which have been reported less frequently, display higher stability and fertility (Chen et al. 2010a, b; Chevre et al. 1991; Quiros et al. 1987; Wang et al. 2006; Zhang et al. 2013). To the best of our knowledge, our report is the first to describe a B. carinata-rapa disomic chromosome addition line. The transfer of the B. rapa A08 chromosome into the carinata genome is clearly demonstrated by the results of the Brassica 90K Illumina Infinium™ SNP genotyping array and further supported.
by the molecular cytogenetic analysis. Thus, considering the relatively unambiguous chromosome counts based on multiple plants, a stable karyotype of 36 chromosomes can be assumed for the interspecific hybrids, as shown for line VR17-095. The results of the molecular cytogenetic analysis suggest that two of the 36 chromosomes are indeed A-genome chromosomes, for a karyotype of \(2n = 34 + 2A\).

The transfer rate of the \(Br\) gene can be used to directly extrapolate the transmission rate of chromosome A08 as \(FAE\) is located on this chromosome (Fourmann et al. 1998; Delourme et al. 2006). It is well known that chromosome transmission rates are generally higher through female gametes than via male gametes (Budahn et al. 2006; Multani et al. 1994; Singh et al. 1998; Zhang et al. 2013); therefore, the focus of this study was on determining the transmission rate of chromosome A08 for male gametes by producing microspore-derived progeny and hybrid offspring by conducting crosses with a CMS \(B.\) \(carinata\) line. Both experimental approaches yielded rates of transmission of over 98%, suggesting virtually stable introduction of A08 to the \(B.\) \(carinata\) genome. In comparison, \(B.\) \(napus\)-\(B.\) \(nigra\) disomic chromosome addition lines showed transmission rates for different nigra chromosomes between 50 and 100% (Chevre et al. 1991), indicating that the degree of transmission can vary depending on the species and chromosome under investigation.

A search on Ensembl Plants (https://plants.ensembl.org/index.html) revealed that chromosome A08 from \(B.\) \(rapa\) harbors additional genes that play a role in lipid metabolism, such as desaturases \(FAD4\) and \(FAD6\) as well as an acyltransferase and \(LPAT5\) (lyso-phosphatidic acid acyltransferase 5). However, it appears that these genes do not have a major impact on the fatty acid profile in the \(fad2B/FAE\) \(B.\) \(carinata\) disomic A-chromosome addition lines as the seed oil composition phenotype of these lines is consistent with that of \(FAE1\) over expression lines in other plants (Kunst 1997; Mietkiewska et al. 2004). It is also worth noting that \(FAD2\) and \(FAD3\), the major genes involved in the synthesis of polyunsaturated fatty acids (Beisson et al. 2003), are located on chromosomes A03, A04 and A05, and not on A08.

In conclusion, this study has shown that the development of super-high erucic acid Ethiopian mustard is possible through traditional interspecific hybridization. To our knowledge, the results presented herein currently represent the highest accumulation of erucic acid achieved in \(B.\) \(carinata\) seeds. Although the lines we developed are not yet suitable for introgression into elite \(B.\) \(carinata\) breeding lines for cultivar development, because of the lack of completely stable introgression of the A08 chromosome, our results nevertheless make \(B.\) \(carinata\) an increasingly attractive alternative to HEA \(B.\) \(napus\) as a feedstock crop for industrial applications. In order to achieve a 100% stability of the \(FAE\) introgression, an attempt to introgress the \(BrFAE\) gene into the C genome through non-homologous recombination between A08 and a \(B.\) \(carinata\) C chromosome using intraspecific hybridization between the \(fad2B/FAE\) \(B.\) \(carinata\) disomic A-chromosome addition lines and resynthesized \(B.\) \(carinata\) is ongoing. Utilizing available \(B.\) \(rapa\) whole genome sequence information, a collection of A08-specific markers can be developed and, in conjunction with the existing \(FAE\) and \(fad2B\) markers, will allow for the selection of promising lines while reducing linkage drag.

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**Author contributions statement** KCF, VR and CE conceived the research plans; VR designed and performed the experiments; CE designed the field experiment; KCF, VR and CE supervised the experiments; ASM and RG designed and performed the molecular cytogenetics analyses; VR and CE analyzed the data; VR and CE wrote the article with input from ASM; all authors contributed to, read and approved the final version of the article.

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**Data availability** All datasets generated for this study are included in the article/supplementary material.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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