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ORIGINAL PAPER

Development of low-linolenic acid *Brassica oleracea* lines through seed mutagenesis and molecular characterization of mutants

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Abstract Designing the fatty acid composition of *Brassica napus* L. seed oil for specific applications would extend the value of this crop. A mutation in *Fatty Acid Desaturase 3* (*FAD3*), which encodes the desaturase responsible for catalyzing the formation of α-linolenic acid (ALA; $18{:}3^{cis\Delta 9,12,15}$), in a diploid *Brassica* species would potentially result in useful germplasm for creating an amphidiploid displaying low ALA content in the seed oil. For this, seeds of *B. oleracea* (CC), one of the progenitor species of *B. napus*, were treated with ethylmethane-sulfonate to induce mutations in genes encoding enzymes involved in fatty acid biosynthesis. Seeds from 1,430 M_2 plants were analyzed, from which M_3 seed families with 5.7–6.9 % ALA were obtained. Progeny testing and selection for low ALA content were carried out in M_3–M_7 generations, from which mutant lines with <2.0 % ALA were obtained. Molecular analysis revealed that the mutation was due to a single nucleotide substitution from G to A in exon 3 of *FAD3*, which corresponds to an amino acid residue substitution from glutamic acid to lysine. No obvious differences in the expression of the *FAD3* gene were detected between wild type and mutant lines; however, evaluation of the performance of recombinant Δ-15 desaturase from mutant lines in yeast indicated reduced production of ALA. The novelty of this mutation can be inferred from the position of the point mutation in the C-genome *FAD3* gene when compared to the position of mutations reported previously by other researchers. This *B. oleracea* mutant line has the potential to be used for the development of low-ALA *B. napus* and *B. carinata* oilseed crops.

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Introduction

The traditional fatty acid (FA) composition of canola (*Brassica napus*, AC genomes) seed oil is mainly represented by palmitic (C16:0, 3–4 %), stearic (C18:0, 1–2 %), oleic ($C18{:}1^{cis\Delta 9}$, 55–60 %), linoleic (LA; $C18{:}2^{cis\Delta 9,12}$; 20–22 %), and α-linolenic (ALA; $C18{:}3^{cis\Delta 9,12,15}$; 10–12 %) FAs. This composition makes canola oil one of the healthiest vegetable oils for human nutrition (Ackman 1990). However, for specific applications, designer oils with altered FA compositions can be desirable (see Scarth and Tang 2006 for review). For example, oil with low levels of ALA and/or high levels of oleic acid exhibits increased oxidative stability (Eskin et al. 1989; Neff et al. 1994; Petukhov et al. 1999; Merrill et al. 2008; see Orthoefer 2005 for review) as well as improved odor under frying conditions (Prévôt et al. 1990) compared to traditional canola oil. ALA is synthesized from oleic acid through desaturation of oleic acid to LA, and LA to ALA, by membrane-bound desaturases encoded by *Fatty Acid Desaturase 2* (*FAD2*) and *Fatty Acid Desaturase 3* (*FAD3*) genes, respectively (Ohlrogge and Browse 1995). Therefore, it would follow that knockdown of the activity of the *FAD3* gene through mutation would yield lower ALA content in seed oil.

It is well established that *B. napus* evolved from the diploid progenitor species *B. rapa* (A genome) and *B. oleracea* (C genome). These two Brassica genomes (A and C) evolved from an *Arabidopsis*-like ancestral prototype

through genome duplication, rearrangements, chromosome fusion, and/or fission events (Lagercrantz 1998; Lagercrantz and Lydiate 1996; Lysak et al. 2005). Therefore, multiple copies of genes involved in FA biosynthesis are often found in each of these genomes. For example, Scheffer et al. (1997) reported that each of the A and C genomes carry 4–6 copies of the *FAD2* gene and 6–8 copies of the *FAD3* gene, which implies that, theoretically, *B. napus* may carry up to 8–12 and 12–16 copies of these two genes, respectively. Therefore, improvement of a trait in *B. napus* is often far more complex than in its diploid progenitor species, as genes from both diploid genomes encode enzymes that contribute to FA/oil biosynthesis in the amphidiploid species (Rahman 2002).

cTraditional plant breeding for the development of a crop cultivar with an improved trait depends on the availability of genetic variation for the trait in crop germplasm. The inductions of genetic variation in a trait through mutagenesis, or creation of a new trait through insertion of a foreign gene, are important approaches for the improvement of crop plants. Genetic engineering of *B. napus* with a lauryl (12:0)-acyl-carrier protein thioesterase gene (*FatB*) cloned from California Bay tree, and the production of a high lauric acid content (>40 % lauric acid) in its seed oil is an excellent example of such manipulation (Volker et al. 1996). However, the development of novel traits through the use of transgenic approaches requires that the resulting engineered plants pass through rigorous regulatory processes prior to approval for commercialization. Conversely, a trait created through traditional mutagenesis requires no regulatory approval or a less stringent regulatory process for approval for commercialization, which is an advantage compared to seeking approval for transgenic crops. Thus, induced mutations, created artificially through the use of mutagenic agents, have been applied to various crop species in order to create genetic variation that does not exist naturally in the species. To date, more than 2,300 mutant cultivars of seed and vegetative crop species have officially been released in various countries (for detailed review, see Jain 2005).

Although the development of an induced mutant line with extreme alterations in FA composition can be very challenging in amphidiploid *Brassica* species due to the involvement of multiple gene copies from its two genomes, this feat is far more achievable in the case of diploid species, which carry fewer gene copies. For example, Auld et al. (1992) obtained a low-ALA (3 % C18:3) mutant *B. rapa* line through an analysis of 4,734 M_2 seeds, while the analysis of approximately eight times more M_2 seeds (39,504) was required for the recovery of a mutant *B. napus* line with 4 % ALA. Today, with the availability of in vitro cell and tissue culture techniques, creation of an amphidiploid species through the assembly of gene(s) from its two diploid progenitor species is not a difficult task (Rahman 2004). So far, induced mutations of the A genome species *B. rapa* have been investigated for the development of FA mutant lines (Auld et al. 1992; Tanhuanpää and Schulman 2002); however, such an attempt has not yet been undertaken with the C genome species *B. oleracea*. This paper reports the development of low-ALA *B. oleracea* lines through induced mutagenesis and molecular characterization of the mutants.

Materials and methods

Plant material

An inbred line of Chinese kale *B. oleracea* var. *alboglabra* (hereafter referred to as *B. oleracea*) was used in this research. The original seeds of this accession were obtained from Lantmännen SW Seed, Sweden, and from this, the inbred line was developed through self-pollination of single plants for six generations. This inbred line is self-compatible and does not require vernalization for flowering (i.e., possesses a spring growth habit). The FA composition of the seed oil of this line is 3 % palmitic (C16:0), 1 % stearic (C18:0), 16 % oleic (C18:1), 13 % linoleic (C18:2), 9 % linolenic (C18:3), 5 % eicosenoic (C20:1), 47 % erucic (C22:1), and 6 % other FAs.

Mutagenic treatments

The mutagenic agent ethyl-methane-sulfonate (EMS) was applied at two different treatment concentrations, 0.75 and 5.0 % (v/v) EMS. The 0.75 % treatment was applied following the method described by Thurling and Depittayanan (1992). For this, seeds were immersed in EMS solution for 12 h at room temperature, and the treated seeds were subsequently washed in running tap water and germinated on sterilized sand in Petri dishes. The 5.0 % treatment was applied as described by Auld et al. (1992). In this case, seeds were soaked in water for 16 h then imbibed in EMS solution for 2 h; treated seeds were subsequently washed in running water and plated in Petri dishes containing sterile sand. Seed germination was recorded 7–10 days after plating, after which time the germinated cotyledonary seedlings were transplanted to pots filled with Sunshine professional Growing Mix (Sunshine Horticulture, Bellevue, WA, USA) and grown in a greenhouse [22/18 ± 3 °C (day/night) with 16 h photoperiod].

Generation of mutant populations and selection of mutants

M_1 plants were self-pollinated manually for the production of M_2 seeds. For this, 10–20 newly opened flowers of each

plant were pollinated with fresh pollen from the same plant, and the entire plant was then covered with a self-pollination bag. The resulting M_2 seeds were seeded in a greenhouse in 4 × 4 × 4.5 cm ($L \times W \times D$) cells, and were transplanted to the field 45 days after seeding prior to FA analysis. The field plot was isolated from other Brassica crops, at the Edmonton Research Station of the University of Alberta, and open-pollinated seeds were harvested at maturity on an individual plant basis for subsequent FA analysis. M_3–M_7 generation plants were grown in a greenhouse, along with the *B. oleracea* parent line (*wt*), and single plant selection for low ALA content was carried out in each generation.

Isolation of *B. oleracea FAD3* (*BoFAD3*) cDNA

Total RNA was isolated from developing siliques at 25–30 days after pollination (DAP) from wild type (wt) *B. oleracea*, as well as two low-ALA mutant *B. oleracea* lines derived from the 5 % EMS treatment, using the Qiagen Plant RNeasy kit (Qiagen, Toronto, ON). Contaminating genomic DNA was removed from the resulting samples using the TURBO DNA-free system (Ambion, Life Technologies Inc., Burlington, ON) and RNA concentrations were determined using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE). First-strand cDNA synthesis was carried out using the SuperScript® III first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen, Life Technologies Inc.) with 1 μg total RNA and an oligo-dT primer. Subsequent PCR assays were performed using Platinum High Fidelity Supermix (Invitrogen) along with degenerate primers BoFAD3F3 (5′-ATG GTT GTY GCT ATG KAC CA-3′) and BoFAD3R5 (5′-RTT GAT TTT RGA TTT GTC AGA AGC-3′), which were designed based on publicly available *FAD3* nucleotide sequences from *B. napus*, *B. juncea*, *B. rapa*, and *Arabidopsis*, and were expected to anneal near the 5′ and 3′ termini of any *FAD3*-like sequences. PCR cycling parameters were as follows: 94 °C for 2 min, 32 cycles of 94 °C for 15 s, 54 °C for 30 s, and 68 °C for 2 min, followed by a final extension at 68 °C for 5 min. The resulting products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The two distinct *FAD3* sequences identified in all three samples tested were termed *BoFAD3-1* and *BoFAD3-2*.

5′ and 3′ rapid amplification of cDNA ends (RACE)

To determine the sequences of the 5′ and 3′ ends of the *BoFAD3-1* and *BoFAD3-2*-coding regions from wt and low-ALA lines, 5′ and 3′ RACE were utilized. In the case of 5′ RACE, SuperScript® III was used for first-strand cDNA synthesis along with 1 μg DNase-treated total RNA derived from wt and low-ALA developing siliques (25–30 DAP) along with the gene-specific primer BoFAD3-1/2R1 (5′-CGA TGG CTT ATT CTC CAA CC-3′), which was designed to anneal to both *BoFAD3-1* and *BoFAD3-2* sequences. The resulting cDNAs were purified and C-tailed, after which time 2.5 μL were utilized in subsequent 25 μL PCRs with Platinum High Fidelity Supermix with primers AAP (Invitrogen), which anneals to the 5′ polyC tail, and BoFAD3-1R2 (5′-GAA CAA TAT GAC CAA CCG CAG TA-3′; *BoFAD3-1*) or BoFAD3-2R2 (5′-GAA GAA TGT GAC CAA CCA CAC TG-3′; *BoFAD3-2*). Nested PCRs were subsequently carried out to improve specificity and increase the amount of product generated using 0.5 μL PCR product as template in a 25 μL reaction along with primers AUAP (Invitrogen) and BoFAD3-1R3 (5′-CCT TGG GCG GCC CAA TAA AGA G-3′; *BoFAD3-1*) or BoFAD3-2 R3 (5′-CCT TGG GCA ACC CAG TAG AGT G-3′; *BoFAD3-2*).

In the case of 3′ RACE, first-strand cDNA synthesis was performed using 1 μg DNase-treated total RNA from wt and low-ALA developing siliques (25–30 DAP) with SuperScript® III and oligo-dT adapter primer AP (Invitrogen). PCR amplifications were subsequently performed using 1 μL cDNA in a 25 μL reaction with primers AUAP (Invitrogen) and BoFAD3-1F1 (5′-CAA TAC CGA CCC ACT TAG TGG AA-3′; *BoFAD3-1*) or BoFAD3-2F1 (5′-CAA TAC CGA TTC ACT TGG TGG AG-3′; *BoFAD3-2*). In each case, purified PCR products were cloned into the pGEM-T vector and sequenced, and full-length *BoFAD3*-coding sequences were derived by combining the previous RT-PCR results along with the RACE data.

Expression and sequence analysis of wt and mutant *BoFAD3*

Semi-quantitative RT-PCR was carried out for both *BoFAD3-1* and *BoFAD3-2* transcripts using 1 μL cDNA from siliques (25–30 DAP) of the wt line and two low-ALA mutant lines, generated as described above. Primers BoFAD3F3c (5′-GAA CGG AGA TTC CAA GGA CG-3′) and BoFAD3R5 were utilized to amplify a 1,108-nt *BoFAD3-1*-specific product, while BoFAD3F3b (5′-AGA TTC CGG TGC CCG GAA GG-3′) and BoFAD3R5 were utilized to amplify a 1,110-nt *BoFAD3*-2-specific product. In addition, a 677-nt putative *BoPP2AA3* transcript (GenBank accession number DK472124) was amplified as an internal control using primers BoPP2AA3F1 (5′-GAC CGA GGA GCC GCT TTA CC-3′) and BoPP2AA3R1 (5′-GAG CAG CAC AAC CCT CAA CC-3′). In each case, Platinum High Fidelity Supermix was utilized for amplification in a final volume of 25 μL. The conditions for PCR amplification of *BoFAD3*-specific cDNA were 94 °C for 2 min, 28 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C

for 1.5 min, with a final extension of 68 °C for 7 min. The same general parameters were used to amplify *BoPP2AA3*-specific fragments, with the exception of the annealing temperature, which was 58 °C, and the extension time, which was 1 min. PCR products were resolved on 1 % agarose gels and visualized with SYBR Safe (Invitrogen).

To determine whether any nucleotide differences were apparent within the coding regions of the wt and mutant *BoFAD3* sequences, RT-PCR products were cloned into pGEM-T and sequenced. In the case of *BoFAD3-1* sequences, ten wt clones and nine mutant clones (from two separate lines) were sequenced. In the case of *BoFAD3-2* sequences, four wt clones and eight mutant clones (from two separate lines) were sequenced. Sequences were subsequently aligned to each other, as well as previously identified *FAD3* sequences from the C genome of *B. napus*, using CLUSTALW.

Heterologous expression of wt and mutant *BoFAD3-1* in yeast

To determine the relative activities of wt and mutant BoFAD3-1 proteins, full-length coding regions of each were amplified from previously generated cDNA using the high-fidelity Pfx polymerase (Invitrogen) along with primers BoFAD3-1F1Y (5′-GCG ATG GTT GTC GCT ATG GAC-3′) and BoFAD3-1R1Y (5′-AGG TTA ATT GAT TTT GGA TTT GTC-3′), which were designed to specifically amplify *BoFAD3-1*. The resulting PCR products were inserted downstream of the *GAL1* promoter in the yeast expression vector, pYES2.1 TOPO®TA (Invitrogen), according to the manufacturer's recommendations and were confirmed by sequencing in each case. *Saccharomyces cerevisiae* strain SCY62 (*MAT* a *ADE2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was transformed with expression vectors containing the wt and mutant *BoFAD3-1*-coding regions, as well as empty vector, respectively, using the lithium acetate method. To induce expression of the heterologous *BoFAD3-1* genes, three separate colonies bearing each vector, including the empty vector control, respectively, were first utilized to generate yeast pre-cultures, which were incubated at 30 °C for 2 days in minimal media supplemented with 2 % raffinose and lacking uracil. These cultures were then used to inoculate 2 × 80 mL of induction media (minimal media containing 2 % galactose and 1 % raffinose, but lacking uracil) to an OD_{600} of 0.2. In each case, one of the two cultures was fed with 150 μM LA (C18:2 in ethanol) along with 0.1 % tyloxapol, while the other was supplemented with the same volume of ethanol and tyloxapol, but no FA. The cultures were then grown at 19 °C with shaking at 225 rpm for 3 days.

FA analysis

Fatty acid analysis of seed oil was conducted with mature bulk seed (0.1–0.25 g) harvested from M_2 to M_7 generation plants. Seeds were ground in *N*-pentane in 50 ml tubes, centrifuged at 1,500 rpm for 10 min, and the supernatant was transferred to 10 × 75 mm glass tubes. The *N*-pentane was evaporated, leaving behind extracted oil. Extracted oil was methylated to produce FA methyl esters, which were analyzed using an Agilent 6890N gas chromatograph to determine the FA profile. Analyses were carried out in the Analytical Laboratory of the Canola Program of the University of Alberta. This laboratory is certified by the Canadian Grain Commission for this type of analysis.

To determine the FA compositions of transformed yeast, each 80 mL culture was divided into two, pelleted by centrifugation, and washed twice with water. Total lipids were isolated by first lysing the yeast by vortexing in 2 mL methanol with 0.5 mm glass beads (Biospec Products Inc., Bartlesville, OK), and then extracting with 4 mL chloroform containing 0.05 mg triheptadecanoin (Nu-Chek Prep Inc., Elysian, MN) as an internal standard. The resulting lipids were dried under N_2 and were then transmethylated by incubating in 1 mL 5 % sodium methoxide at room temperature for 30 min. Reactions were quenched through the addition of 1 mL 0.9 % NaCl, and FA methyl esters were extracted twice with 2 mL hexane. The resulting extracts were dried under N_2, resuspended in 200 μL isooctane and analyzed by gas chromatography/mass spectrometery (GC/MS) using an Agilent 6890N gas chromatograph with Agilent 5975 Inert Mass Selective Detector (Agilent Technologies Canada Inc., Mississauga, ON). Chromatographic separation was carried out using a DB-23 capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA) with an initial temperature of 165 °C for 4 min, which was then raised at 10 °C/min to 180 °C, held for 5 min, then raised at 10 °C/min to a final temperature of 230 °C. The inlet was operated in split mode (10:1 split ratio, 1 μL injection) at a temperature of 290 °C, with a constant helium flow of 10.9 mL/min. For mass spectra analyses, the data was acquired in scan mode with a range 30–350 amu. Relative percentages of ALA were derived from peak areas. Conversion rates of FA substrate to product were calculated by dividing the weight percent product by the sum of the weight percent substrate and product, then multiplying by 100.

Statistical analyses

Scattered diagrams and statistical analyses including mean, variance, standard error, confidence intervals and *t* tests were calculated using the EXCEL program. For paired two

tailed t tests, differences were considered significant at $P \leq 0.01$.

Results

EMS treatment and generation of M_1 plants

A total of 1,000 seeds were treated with 0.75 and 5 % EMS. The viability of untreated *B. oleracea* (control) seeds was 98.3 %. In the case of seeds that had undergone the 0.75 % treatment, 85.5 % of the treated seeds germinated, while more than 95 % of seeds derived from the 5 % treatment were viable (Table 1). A total of 250 and 586 M_1 plants derived from 0.75 and 5 % treatments, respectively, were grown. The majority of resulting mutant plants produced only small amounts of visible pollen compared to untreated *B. oleracea*; therefore, all M_1 plants were self-pollinated manually for production of M_2 seeds. Following self-pollination, approximately 30 % of the seeded plants produced seed. The number of seeds harvested from M_1 plants ranged from 1 to 204. Conversely, 90 % of the untreated *B. oleracea* control plants produced seed following self-pollination. Thus, it was evident that there was a clear effect of EMS treatment on the fertility of resulting M_1 plants.

Selection for low-ALA seed phenotypes in the M_2–M_7 generations

A total of 3,885 M_2 plants derived from 0.75 and 5 % EMS treatments were grown under field conditions, of which 88 % plants produced seeds (Table S1). Mature seeds harvested from 375 and 1,055 M_2 plants from the 0.75 and 5 % treatments, respectively, were used for FA analysis. Scatter diagrams of the two M_2 populations for α-linolenic acid (ALA) plotted against oleic acid are presented in Fig. 1. While the ALA content in the seed oil of untreated *B. oleracea* ranged between 8.4 and 11.6 % with a mean of 10.15 ± 0.14 %, the content of this FA in seeds harvested from 0.75 and 5 % EMS treated M_2 plants varied from 7.9 to 15.7 % (mean 12.01 ± 0.08) and 5.1–15.1 % (mean 10.55 ± 0.05), respectively (Table S1). Approximately 6.7 % (25) and 31.0 % (327) of the 0.75 and 5 % EMS treated M_2 plants, respectively, bore seeds with significantly lower ALA content than wt *B. oleracea* (confidence limit, $CL_{0.05}$ = 9.78–10.52 %). As a result, selection for low ALA content was carried out on the population derived from the 5 % EMS treatment. From this M_2 population, seven M_3 seed families (seeds harvested from M_2 plants) (Table S1) with <7.0 % linolenic acid were selected and grown to maturity. The ALA content in seeds of the M_3 generation varied from 3.3 to 8.4 % (mean 5.55 ± 0.18). This was significantly lower than the wt *B. oleracea* grown alongside this population (Fig. 2; Table S1). Further selection for low-ALA acid content effectively reduced the level of this FA and led to the development of a genetically stable low-ALA line (Fig. 2), with seeds from M_7 lines exhibiting ≤2 % ALA content in contrast to 9 % ALA in wt plants grown under the same environmental conditions (Fig. 2; Table S1 and S2). This reduction in ALA content in the mutant lines also yielded an increase of 5 and 2 % in LA and oleic acid contents, respectively. However, the sum of LA and ALA contents were very similar in the wt and M_7 lines (21.6 vs. 20.2 %) suggesting that mutation of the *FAD3* gene, which is involved in the desaturation of LA to ALA, was responsible for the low ALA phenotype. The contents of other FAs, such as C12:0, C14:0, C16:0, C18:0, C20:0, C20:1, C22:0, C22:1, C24:0, and C24:1, were very similar in the wt and mutant lines (Table S2).

BoFAD3 transcripts from developing siliques of *B. oleracea*

As an initial step in identifying *BoFAD3* cDNA sequences from *B. oleracea*, degenerate primers designed to anneal near the 5′ and 3′ ends of known plant *FAD3*-coding regions were utilized to amplify potential transcripts from RNA derived from *B. oleracea* developing siliques. Two distinct sequences (*BoFAD3-1* and *BoFAD3-2*) that exhibited high similarity to *FAD3*-coding regions from other plant species were isolated. To determine the full-length coding regions of both genes, a combination of 5′ and 3′ RACE were utilized. Comparison of the two full-length 1,143-bp (*BoFAD3-1*) and 1,152-bp (*BoFAD3-2*) sequences

Table 1 Treatment of *Brassica oleracea* var. *alboglabra* seeds with ethyl-methane-sulfonate (EMS) and production of M_2 seeds

EMS treatment	No. seeds treated	No. seeds germinated	% germinated seeds	No. seedlings to soil	No. M_1 plants produced seeds	% M_1 plants produced seeds	No. seeds on M_1 plants		No. M_2 seeds harvested
							Range	Mean ± S.E.	
0.75 %	400	342	85.5	250	70	28.0	1–204	15.1 ± 4.0	1,060
5.0 %	600	586	97.7	586	211	36.0	1–158	15.2 ± 1.8	3,216
Total	1,000	928	92.8	836	281	33.6			4,276
Control	120	118	98.3			90.0[a]			

[a] 10 plants grown to maturity of which nine produced seeds. Number of seeds per plant were ~90

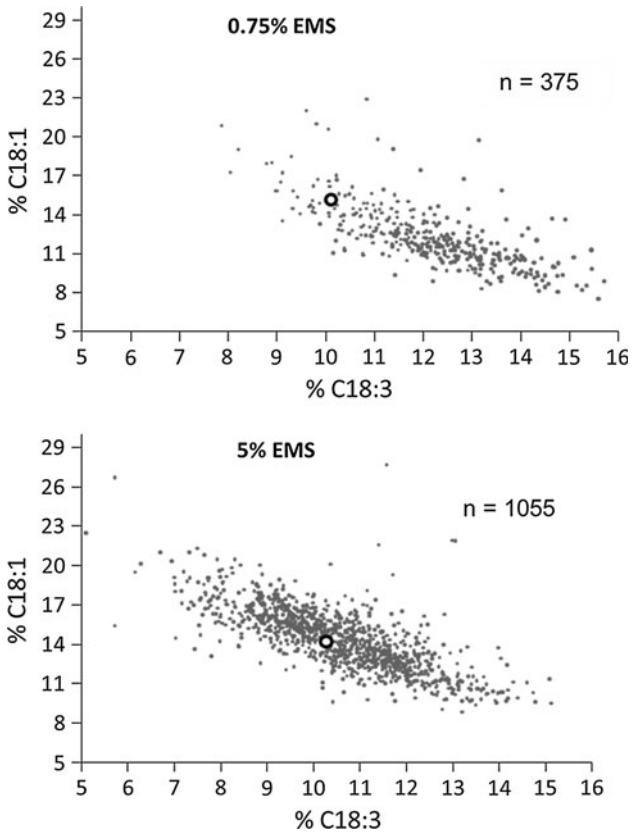

Fig. 1 Scatter diagram for percent α-linolenic acid (C18:3) content plotted against oleic acid (C18:1) in M_2 populations of *Brassica oleracea* var. *alboglabra* generated from seed mutagenized with 0.75 and 5.0 % ethyl-methane-sulfonate (EMS). *Open circle B. oleracea* var. *alboglabra* control

from the wt line (GenBank accession numbers JX866747 and JX866748, respectively) indicated that they exhibited 93.3 % identity at the nucleotide level (Fig. 3). In addition, comparison of each sequence to those previously identified from the C genome of *B. napus* (Yang et al. 2012) demonstrated that while *BoFAD3-1* appeared to be orthologous to *BnaC.FAD3.b* (99.6 % identity at the nucleotide level, compared to 93.3 and 86.2 % identity with *BnaC.FAD3.a* and *BnaC.FAD3.c*, respectively), *BoFAD3-2* appeared to be orthologous to *BnaC.FAD3.a* (99.8 % identity at the nucleotide level, compared to 93.1 and 88.3 % identity to *BnaC.FAD3.b* and *BnaC.FAD3.c*, respectively) (Figs. S1 and S2). Deduced proteins of *BoFAD3-1* and *BoFAD3-2* were 380 and 383 amino acids in length, respectively, and shared 94.5 % amino acid identity (Fig. S3).

Low-ALA mutant lines possess a single point mutation in *BoFAD3-1*

In an attempt to ascertain whether the low-ALA phenotype in mutant lines was associated with one or more mutations within the *BoFAD3-1*- or *BoFAD3-2*-coding regions, wt sequences were compared with those isolated from two low-ALA EMS mutants. Interestingly, while the *BoFAD3-2* sequences were identical in wt and mutant lines, *BoFAD3-1* contained a single nucleotide substitution from G to A (Fig. 4) at position +424 (where +1 corresponds to the first nucleotide of the translational start site) in both mutant lines compared to wt. This site lies within the putative third exon of the *BoFAD3-1* gene. In the deduced proteins, this mutation corresponded to an amino acid substitution from a glutamic acid to lysine residue at position 142 in the amino acid sequence (Fig. 5).

Semi-quantitative RT-PCR was carried out to determine whether expression levels of the *BoFAD3* genes differed between wt and low-ALA mutant lines. For both *BoFAD3-1* and *BoFAD3-2*, the results indicated that there were no obvious differences in their expression levels between wt and mutant lines (Fig. S4).

Mutant *BoFAD3-1* exhibits reduced desaturase activity in yeast compared to wt

To establish whether the mutant *BoFAD3-1* coding region from low-ALA lines encoded a protein with reduced Δ-15 desaturase activity compared to the wt *BoFAD3-1* sequence, both wt and mutant *BoFAD3-1*-coding regions were expressed in *S. cerevisiae*. Supplementation of the transformed yeast cultures bearing wt *BoFAD3-1* with LA led to the production of ALA at an average level of 0.40 % ± 0.13 SD of the total FA content. Conversely, yeast containing the mutant *BoFAD3-1* sequence generated significantly reduced amounts of ALA (0.015 % ± 0.002 SD of the total FA content) when supplemented with LA (Fig. S5). The average conversion rate in cultures expressing wt *BoFAD3-1* was 5.21 % ± 1.81 SD, which was significantly higher than that observed in cultures expressing the mutant *BoFAD3-1* (0.20 % ± 0.03 SD). Conversely, neither yeast transformed with empty vector, nor transformed yeast expressing the two *BoFAD3* variants that were not supplemented with exogenous LA, produced any detectable ALA (Figs. S5; Fig. 6).

Discussion

In the present study, we developed for the first time a low-ALA (approximately 2 % C18:3) *B. oleracea* mutant line via the application of EMS seed mutagenesis. Use of a self-compatible inbred *B. oleracea* var. *alboglabra* line facilitated the selection for low ALA content through self-pollination. Of the two EMS treatments applied, we obtained low-ALA lines from 5.0 % EMS treatment, but not from the 0.75 % treatment. The 0.75 % treatment included seeds

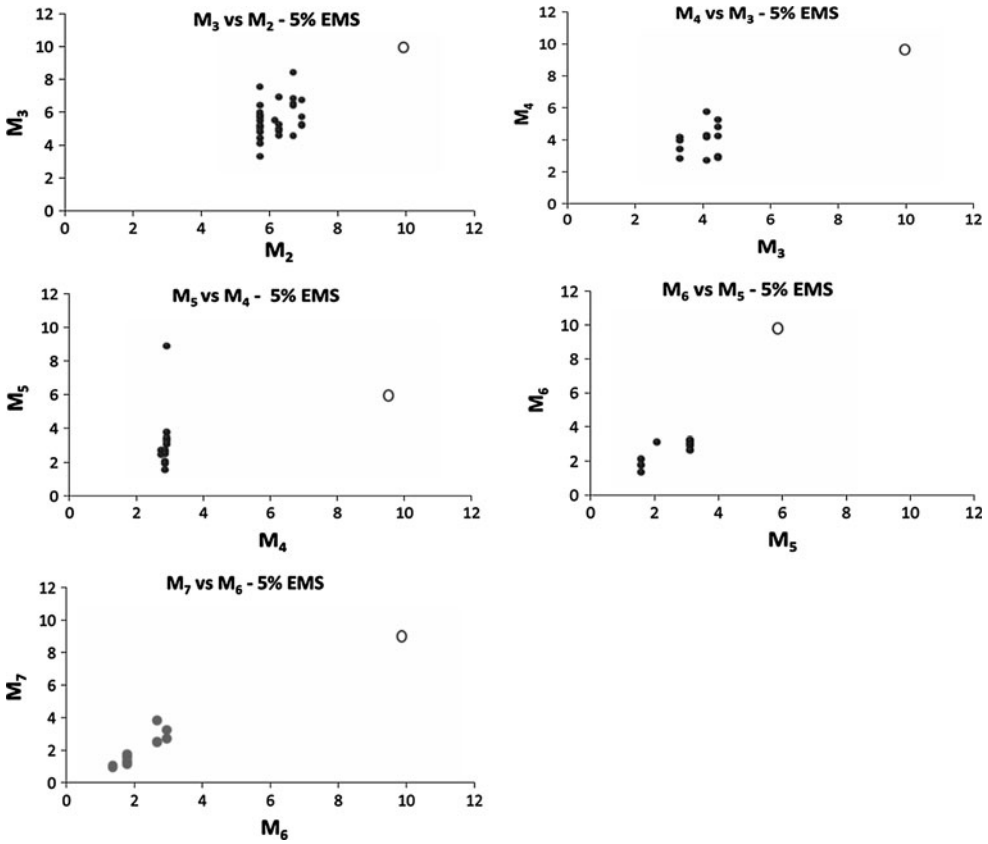

Fig. 2 Distribution of percent α-linolenic acid (C18:3) content in seed oil of M_3 to M_7 generations of *Brassica oleracea* var. *alboglabra* generated from seed mutagenized with 5.0 % ethyl-methane-sulfonate (EMS). *Open circle B. oleracea* var. *alboglabra* control

from 375 M_2 plants, which is a very small size population when it is considered that finding a mutant for a specific trait is similar to finding a 'needle in a haystack'. Auld et al. (1992) developed a low-ALA (3 % C18:3) mutant *B. rapa* line through the screening of 4,734 M_2 seeds. In the present study, however, we obtained low-ALA *B. oleracea* lines through the analysis of seeds from 1,055 M_2 plants followed by selection for this trait in subsequent generations.

Based on bioinformatic analyses and molecular cloning, Yang et al. (2012) identified three predicted copies of *FAD3* in both *B. rapa* and *B. oleracea*, and six homologs in *B. napus*—one copy in each of the A3, A4, A5, and C3 chromosomes and two copies in C4. The genomic sequences of these six genes, as well as their gene structures, indicated a wide range of variation among them; however, they shared a high level of identity at the amino acid sequence level. Phylogenetic analysis of the genes based on their predicted amino acid sequences resulted in the clustering of these six *FAD3* genes into three groups: *FAD3.a* (*BnaA.FAD3.a* of A5 and *BnaC.FAD3.a* of C4), *FAD3.b* (*BnaA.FAD3.b* of A4 and *BnaC.FAD3.b* of C4), and *FAD3.c* (*BnaA.FAD3.c* of A3 and *BnaC.FAD3.c* of C3) (Yang et al. 2012). Of the three *B. napus* C genome *FAD3* gene copies, the *B. oleracea* *BoFAD3-1* and *BoFAD3-2* transcripts identified in our study were most closely related to *BnaC.FAD3.b* and *BnaC.FAD3.a*, respectively (Figs. S1 and S2).

The first low-ALA (approximately 3 % C18:3) mutant *B. napus* line was developed in the 1970s (Rakow 1973; Röbbelen and Nitsch 1975); this mutant was found to carry two mutations in *FAD3* genes from the A and C genomes. These genes encode a microsomal (endoplasmic) Δ^{15} desaturase, which catalyzes the formation of ALA from LA (Jourdren et al. 1996a, b; Barret et al. 1999; Rajcan et al. 1999). Since the development of this mutant, several other researchers have developed additional *B. napus* mutants with similar low ALA content through the application of EMS mutagenesis (Auld et al. 1992; Hu et al. 2006; Spasibionek 2006). For example, Hu et al. (2006) reported that their low-ALA (approximately 3 % C18:3) spring *B. napus* line was due to mutations in two loci located on chromosomes C4 and A4, and that these two mutations were responsible for approximately 52 and 25 % of the total variation, respectively. This C4 locus corresponded to one of the two loci reported by Jourdren et al. (1996b) in the low-ALA mutant line developed by Rakow (1973) and Röbbelen and Nitsch (1975) (Hu et al. 2006). Similarly, Mikolajczyk et al. (2010) reported that the low-ALA (approximately 2 % C18:3) winter *B. napus* line developed by Spasibionek (2006) was also the result of a mutation in two *FAD3* genes, one in the A and the other in the C

Fig. 3 Comparison of *BoFAD3-1-* (*top*) and *BoFAD3-2-* (*bottom*) coding regions from wt *Brassica oleracea* var. *alboglabra*. *Asterisks* indicates identical nucleotide identities

```
wt          ATGGTTGTCGCTATGGACCAGCGTAGCAATGTGAACGGAGATTCCAAGGACGAAAGGTTT
mutant      ATGGTTGTCGCTATGGACCAGCGTAGCAATGTGAACGGAGATTCCAAGGACGAAAGGTTT
            ************************************************************

wt          GATCCGAGCGCACAACCACCGTTTAAGATCGGAGATATAAGGGCGGCGATTCCTAAGCAT
mutant      GATCCGAGCGCACAACCACCGTTTAAGATCGGAGATATAAGGGCGGCGATTCCTAAGCAT
            ************************************************************

wt          TGTTGGGTAAAGAGTCCTTTGAGATCCATGAGCTACGTCGCGAGAGACATTTTCGCCGTC
mutant      TGTTGGGTAAAGAGTCCTTTGAGATCCATGAGCTACGTCGCGAGAGACATTTTCGCCGTC
            ************************************************************

wt          GTGGCTCTGGCCGTCGCCGCCGTGTATTTTGATAGCTGGTTCTTCTGGCCTCTTTATTGG
mutant      GTGGCTCTGGCCGTCGCCGCCGTGTATTTTGATAGCTGGTTCTTCTGGCCTCTTTATTGG
            ************************************************************

wt          GCCGCCCAAGGAACCCTTTTCTGGGCCATCTTCGTACTCGGCCACGACTGTGGACATGGG
mutant      GCCGCCCAAGGAACCCTTTTCTGGGCCATCTTCGTACTCGGCCACGACTGTGGACATGGG
            ************************************************************

wt          AGCTTCTCAGACATTCCTCTTCTGAATACTGCGGTTGGTCATATTCTTCATTCCTTCATT
mutant      AGCTTCTCAGACATTCCTCTTCTGAATACTGCGGTTGGTCATATTCTTCATTCCTTCATT
            ************************************************************

wt          CTCGTTCCATACCATGGTTGGAGAATAAGCCATCGGACACACCACCAGAACCATGGCCAT
mutant      CTCGTTCCATACCATGGTTGGAGAATAAGCCATCGGACACACCACCAGAACCATGGCCAT
            ************************************************************

wt          GTTGAAAACGACGAGTCTTGGGTTCCGTTGCCAGAAAAATTATACAAGAATTTGTCCCAC
mutant      GTTAAAAACGACGAGTCTTGGGTTCCGTTGCCAGAAAAATTATACAAGAATTTGTCCCAC
            ***  ********************************************************

wt          AGTACACGGATGCTCAGATACACTGTCCCTCTCCCCATGCTCGCTTACCCTCTCTATCTG
mutant      AGTACACGGATGCTCAGATACACTGTCCCTCTCCCCATGCTCGCTTACCCTCTCTATCTG
            ************************************************************

wt          TGGTACAGAAGTCCTGGTAAAGAAGGGTCACATTATAACCCATACAGTAGTTTATTTGCC
mutant      TGGTACAGAAGTCCTGGTAAAGAAGGGTCACATTATAACCCATACAGTAGTTTATTTGCC
            ************************************************************

wt          CCAAGCGAGAGAAAGCTTATTGCAACTTCAACTACTTGCTGGTCGATCGTGTTGGCCACT
mutant      CCAAGCGAGAGAAAGCTTATTGCAACTTCAACTACTTGCTGGTCGATCGTGTTGGCCACT
            ************************************************************

wt          CTTGTTTATCTATCATTCCTCGTTGGTCCAGTCACAGTTCTAAAAGTCTATGGTGTTCCT
mutant      CTTGTTTATCTATCATTCCTCGTTGGTCCAGTCACAGTTCTAAAAGTCTATGGTGTTCCT
            ************************************************************

wt          TACATTATCTTTGTAATGTGGTTGGACGCTGTCACGTACTTGCATCATCATGGTCACGAT
mutant      TACATTATCTTTGTAATGTGGTTGGACGCTGTCACGTACTTGCATCATCATGGTCACGAT
            ************************************************************

wt          GATAAGTTGCCTTGGTACAGAGGCAAGGAATGGAGTTATTTACGTGGAGGATTAACAACT
mutant      GATAAGTTGCCTTGGTACAGAGGCAAGGAATGGAGTTATTTACGTGGAGGATTAACAACT
            ************************************************************

wt          GTTGATAGAGATTACGGGATCTTCAACAACATTCATCACGATATTGGAACTCACGTGATC
mutant      GTTGATAGAGATTACGGGATCTTCAACAACATTCATCACGATATTGGAACTCACGTGATC
            ************************************************************

wt          CATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTCGATGCCACGAAAGCAGCTAAA
mutant      CATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTCGATGCCACGAAAGCAGCTAAA
            ************************************************************

wt          CATGTGTTGGGAAGATACTACAGAGAACCAAAGACGTCAGGAGCAATACCGACCCACTTA
mutant      CATGTGTTGGGAAGATACTACAGAGAACCAAAGACGTCAGGAGCAATACCGACCCACTTA
            ************************************************************

wt          GTGGAAAGTTTGGTGGCAAGTATTAAGAAAGATCATTACGTCAGTGACACTGGTGATATT
mutant      GTGGAAAGTTTGGTGGCAAGTATTAAGAAAGATCATTACGTCAGTGACACTGGTGATATT
            ************************************************************

wt          GTCTTCTACGAGACAGATCCAGATCTCTACGTTTATGCTTCTGACAAATCCAAAATCAAT
mutant      GTCTTCTACGAGACAGATCCAGATCTCTACGTTTATGCTTCTGACAAATCCAAAATCAAT
            ************************************************************

wt          TAA
mutant      TAA
            ***
```

Fig. 4 Comparison of *BoFAD3-1* coding regions from wt (*top*) and low α-linolenic acid (C18:3) mutant (*bottom*) lines of *Brassica oleracea* var. *alboglabra*. *Asterisks* indicates identical nucleotide identities

genome, where the C genome mutation occurred in exactly the same gene as that reported by Hu et al. (2006). Yang et al. (2012) also reported a low-ALA spring *B. napus* mutant line (approximately 3 % ALA), which was obtained from Lantmännen SW Seed, Sweden. Molecular mapping of this line identified four QTL on chromosomes A4, A5,

```
wt       MVVAMDQRSNVNGDSKDERFDPSAQPPFKIGDIRAAIPKHCWVKSPLRSMSYVARDIFAV
mutant   MVVAMDQRSNVNGDSKDERFDPSAQPPFKIGDIRAAIPKHCWVKSPLRSMSYVARDIFAV
         ************************************************************

wt       VALAVAAVYFDSWFFWPLYWAAQGTLFWAIFVLGHDCGHGSFSDIPLLNTAVGHILHSFI
mutant   VALAVAAVYFDSWFFWPLYWAAQGTLFWAIFVLGHDCGHGSFSDIPLLNTAVGHILHSFI
         ************************************************************

wt       LVPYHGWRISHRTHHQNHGHVENDESWVPLPEKLYKNLSHSTRMLRYTVPLPMLAYPLYL
mutant   LVPYHGWRISHRTHHQNHGHVKNDESWVPLPEKLYKNLSHSTRMLRYTVPLPMLAYPLYL
         ********************:***************************************

wt       WYRSPGKEGSHYNPYSSLFAPSERKLIATSTTCWSIVLATLVYLSFLVGPVTVLKVYGVP
mutant   WYRSPGKEGSHYNPYSSLFAPSERKLIATSTTCWSIVLATLVYLSFLVGPVTVLKVYGVP
         ************************************************************

wt       YIIFVMWLDAVTYLHHHGHDDKLPWYRGKEWSYLRGGLTTVDRDYGIFNNIHHDIGTHVI
mutant   YIIFVMWLDAVTYLHHHGHDDKLPWYRGKEWSYLRGGLTTVDRDYGIFNNIHHDIGTHVI
         ************************************************************

wt       HHLFPQIPHYHLVDATKAAKHVLGRYYREPKTSGAIPTHLVESLVASIKKDHYVSDTGDI
mutant   HHLFPQIPHYHLVDATKAAKHVLGRYYREPKTSGAIPTHLVESLVASIKKDHYVSDTGDI
         ************************************************************

wt       VFYETDPDLYVYASDKSKIN
mutant   VFYETDPDLYVYASDKSKIN
         ********************
```

Fig. 5 Amino acid alignment of *BoFAD3-1* from wt and mutant *B. oleracea* var. *alboglabra* lines. *Asterisks* indicates identical amino acid identities; *colon* denotes an amino acid substitution

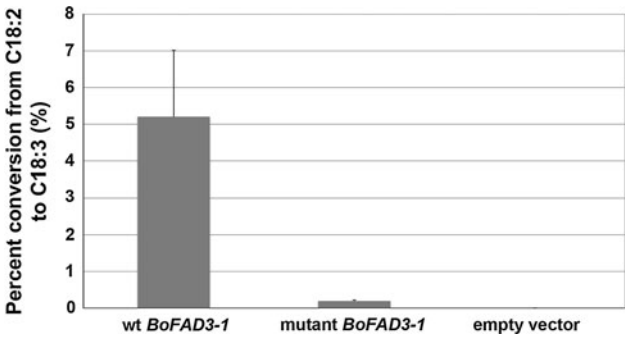

Fig. 6 Activity of wt versus mutant BoFAD3-1 in *S. cerevisiae*. FAMEs derived from three independent colonies grown under inducing conditions and supplemented with 150 μM C18:2 bearing either wt BoFAD3-1, mutant BoFAD3-1 or empty vector, respectively, were analyzed by GC–MS to determine desaturase activity in each case. *Blocks* represent the mean percent conversion from C18:2 to C18:3 of three biological and two technical replicates. *Bars* denote standard deviations

A6, and C4, and these QTL collectively explained >90 % of the total phenotypic variation in ALA content. The QTL on C4 accounted for >60 % of the phenotypic variation and the A4 QTL was responsible for 29 % of the variation. Interestingly, a G to A mutation in the *FAD3* gene of the C4 locus was found to be the same as the mutation identified by Hu et al. (2006) and Mikolajczyk et al. (2010), while the C to T mutation in A4 was the same as the mutation unveiled by Mikolajczyk et al. (2010). On the other hand, based on *Arabidopsis thaliana*-based candidate gene probes, Scheffer et al. (1997) were able to map five *FAD3* loci on chromosomes A3, A4, A5, and C4, where C4 carried two gene loci; and another *FAD3* gene likely to be present on C3 (Smooker et al. 2011). Thus, it is apparent that while only a single C genome *FAD3* gene has so far been modified through the mutagenesis of *B. napus*, this species almost certainly harbors multiple copies of this gene (Scheffler et al. 1997; Yang et al. 2012). In light of this, the creation of lines bearing mutations in any other additional C genome *FAD3* gene would likely allow the development of *B. napus* cultivars with seed oil containing <3 % ALA.

Sequence analysis and alignment of the *FAD3* gene of the C4 locus of wild type *B. napus* and the mutants reported by Hu et al. (2006), Mikolajczyk et al. (2010), and Yang et al. (2012) revealed that the G to A base substitution in the low-ALA acid allele occurred at the 5′ splice site of the sixth intron of this gene; which resulted in impaired splicing and caused the retention of the entire sixth intron in the mature mutant transcript (Hu et al. 2007). The sixth intron contained stop codons in all three possible reading frames. Thus, the retention of this intron resulted in early termination and synthesis of a truncated and defective *FAD3* polypeptide, blocking the desaturation of LA to ALA, and resulting in a low-ALA phenotype. In contrast, the mutation resulting in the low-ALA phenotype in *B. oleracea* developed in the present study occurred within the putative third exon of the *BoFAD3-1* gene, which corresponds to an amino acid substitution from a glutamic acid to lysine residue (Fig. 5) that significantly reduced the activity of the enzyme (Fig. S5 ; Fig.6). Thus, the mutation resulting in the low-ALA *B. oleracea* phenotype identified

in this study appears to occur within the same C genome *FAD3* copy (*BnaC.FAD3.b*) as described above, but it was derived from a novel mutation. Further confirmation of this, as well as the phenotypic effect of this novel mutant allele when compared with the previously reported mutant allele of *BnaC.FAD3.b*, is in progress through cross-breeding of this low-ALA *B. oleracea* line with the low-ALA *B. napus* line carrying the mutant allele of the C genome *FAD3* gene (*BnaC.FAD3.b*). The low-ALA *B. oleracea* mutant line developed in the current study, however, has the potential to be used as an alternative to the previously reported C genome mutant for reduction of seed ALA content in *B. napus* cultivars.

In addition to the potential use of the C genome *Bo-FAD3-1* mutant for genetic improvement of *B. napus*, there are also other Brassica species that could also benefit from its use. For example, while *B. napus*, *B. juncea*, and *B. rapa* are widely grown as oilseed crops, *B. carinata* is traditionally grown in Ethiopia as a leaf vegetable and as an oilseed. This latter species is known to be highly tolerant to both heat and drought, and therefore could be a potential oilseed crop for dry areas such as the Canadian prairies, rain-fed Mediterranean areas, and India. While zero or low-erucic acid seed oil genotypes of this species have already been developed (Alemayehu and Becker 2001; Rakow and Getinet 1998; Velasco et al. 1995), their seed oil generally contains $\geq$10 % ALA. Since *B. carinata* shares the B genome of *B. nigra* and the C genome of *B. oleracea*, the low-ALA mutant *B. oleracea* lines developed in this research also have the potential to be used for the development of low-ALA seed oil from *B. carinata*.

In conclusion, we have created a novel mutant allele of the *FAD3* gene, which has the potential to be used in the breeding of low ALA *Brassica* oilseed crops carrying the C genome. While ALA has been found to play an important role in pollen development (McConn and Browse 1996), studies have not yet been carried out to ascertain whether the low-ALA trait ($\sim$3 %) present in a number of *B. napus* cultivars is associated with any impairment in pollen development. Therefore, while the future aim is to virtually eliminate ALA content from *Brassica* seed oil through the creation of mutations in additional *FAD3* gene copies, further investigations would be required to determine whether this reduction would cause any detrimental effect on microsporogenesis and pollen development.

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