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Simultaneous over-expressing of an acyl-ACP thioesterase (FatB) and silencing of acyl-acyl carrier protein desaturase (SAD) by artificial microRNAs increases saturated fatty acid levels in *Brassica napus* seeds

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Running Title: Increasing saturated fatty acids in rapeseed.

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Summary

No temperate oilseeds crops are available that produce oil with a high saturated fatty acid content. To achieve such a profile, *Brassica napus* cv. DH12075 was engineered by simultaneous seed specific over-expression of a native fatty acyl-ACP thioesterase B [BnFATB(2)] and artificial microRNA mediated down-regulation of eight endogenous genes encoding putative stearoyl-ACP desaturases (BnSADs). Semi-quantative RT-PCR analysis of transformed lines showed that the *BnFATB(2)* gene was highly over-expressed and the 8 putative SAD genes were strongly down-regulated in developing seed demonstrating the successful application of microRNA as a tool for down-regulation of genes in the allotetraploid plant *B. napus*. Analysis of seed triacylglycerol (TAG) composition revealed that all lines contained high levels of palmitic acid (16:0) and moderately increased levels of stearic acid (18:0). Total saturated fatty acid content was increased from 7.4 % in the control to 37.3-45.6 % in the transformed lines, with *FatB* over-expression as the dominant trait. A two fold increase in 16:0 was observed in seed polar lipids. The melting point of oil from mature seeds was increased from -10 °C in DH12075 to 15 °C in the line with the highest saturated fatty acid content. TAG composition showed a shift from predominantly C54 TAG to C50 and C52 TAGs enriched in palmitic acid. Seedling establishment at low temperature was compromised in lines with high saturated fatty acid content. Results suggested that transcript encoding stearoyl-ACP desaturase in developing *B. napus* seeds is present in considerable excess of the level required for efficient desaturation of 18:0.

Introduction

As a result of their fatty acid composition, triacylglycerol (TAG) oils from temperate oilseeds crops are liquid, a property that limits their direct use in food applications such as margarine and baking shortenings where solid fats are required. For conversion from liquid oil to a spreadable or solid fat, these oils must either be blended or inter-esterified with other oils rich in saturated fatty acids, such as palm oil *(Elaeis* spp.), coconut oil *(Cocos nucifera*), hydrogenated vegetable oil or tallow (Chrysan 2005). These approaches all have associated problems and limitations. For example, inter-esterification or blending with oils derived from tropical sources, particularly palm oil, is complicated by public perception concerning the sustainability and ecological impact of production (Laurance et al., 2010). Use of hydrogenated oil can lead to the unintentional introduction of undesirable *trans* fatty acids (TFAs), resulting from incomplete hydrogenation or double bond isomerization (Sommerfeld 1983). Under current dietary guidelines, it is recommended that consumption of TFAs from hydrogenated oils should be avoided (Hunter 2006). A potential solution is to modify a temperate oilseed crop, through genetic engineering, to produce oil with a higher saturated fatty acid content. This oil could be used directly, or as a source of saturates for blending/inter-esterification. For *Brassica napus* (canola/rapeseed) a number of attempts have been made to achieve this goal resulting in oils enriched in specific fatty acids including medium chain fatty acids, palmitic (hexadecanoic acid, 16:0) and stearic acids (octadecanoic acid, 18:0) (Stoll et al., 2005).

The pathways of fatty acid biosynthesis and seed TAG assembly in oilseed species have been extensively studied and recent reviews include those of Baud and Lepiniec (2010) and Bates et al., (2013). In summary, *de-novo* fatty acid biosynthesis occurs in the plastids in developing seeds. Synthesis is initiated by the condensation of acetyl-coenzyme A (acetyl-CoA) and malonyl-acyl carrier protein (malonyl-ACP), a multistep process yielding a 4-carbon acyl-ACP. In *B. napus*, repeated cycles of 2-carbon elongation results in the synthesis of palmitoyl-ACP (16:0-ACP). Release of 16:0 from ACP, catalyzed by an acyl-ACP thioesterase, with subsequent export from the plastid and activation to 16:0-CoA, makes palmitic acid available for cytosolic processes including TAG assembly. Alternatively, newly formed 16:0-ACP can undergo a further 2 carbon elongation to stearoyl-ACP (18:0-ACP), with the condensation step being catalyzed by the enzyme 3-ketoacyl-ACP synthase II (KASII). Desaturation between carbons 9 and 10, catalyzed by stearoyl-ACP desaturase (SAD) then yields oleoyl-ACP (*cis*-9 octadecenoyl-ACP; 18:1-ACP). For further desaturation by the endoplasmic reticulum fatty acid

desaturases, and for TAG assembly, 18:1 is released from ACP by a second acyl-ACP thioesterase with export from the plastid and activation to 18:1-CoA. As described by Stoll and co-workers (2005), acyl-ACP thioesterases, 3-ketoacyl-ACP synthase enzymes (KAS) and SAD have all been targeted in various studies aimed at increasing the saturated fatty acid composition of *B.napus* seed oil. As the terminal step in the fatty acid biosynthesis pathway, acyl-ACP thioesterases determine the chain length of acyl groups leaving the plastid for further metabolism in the cytosol. Higher plant acyl-ACP thioesterases can be divided into two distinct classes based on amino acid sequence. Referred to as FatA and FatB, these thioesterases primarily hydrolyse 18:1-ACP and C8-C16 saturated acyl-ACPs respectively (Jones et al., 1995, Salas and Ohlrogge 2002, Mayer and Shanklin 2007). The acyl specificity of FatB thioesterases has been used to advantage to engineer medium chain saturated fatty acid production and palmitic acid levels in *B. napus* (Voelker et al., 1992, Jones et al., 1995, Dehesh et al., 1996). Thioesterase mediated engineering of 18:0 in *B. napus* was more problematic, but was achieved by expression of a *Garcinia mangostana* (mangosteen) FatA1 acyl-ACP thioesterase, modified by site directed mutagenesis to increase enzyme activity towards 18:0-ACP. Resulting plants accumulated up to 20% stearic acid in their seed oil compared to approximately 2% in control lines (Facciotti et al., 1999). Manipulation of KAS activity in *B. napus* to achieve increased levels of palmitic acid has been less successful. Seed specific expression of a KASIII (catalyzing the first condensation step of fatty acid biosynthesis) from *Cuphea hookeriana* increased 16:0 levels from 4% to almost 9%, but severely impacted lipid synthesis and oil content (Dehesh et al., 2001). Targeting desaturation of 18:0 to increase saturated fatty acid levels was one of the first examples of the successful engineering of a seed oil profile. Antisense mediated suppression of SAD was used to increase 18:0 in *B. napus* from 2% to 40% (Knutson et al., 1992) and from 3.7% to 32% in a more recent report (Zarhloul et al., 2006). Successful enhancement of saturated fatty acid content has also been reported for other oilseed species. For example, over-expression of endogenous *FatB1*, or down regulation of KASII has been used to increase palmitic acid levels in *Arabidopsis thaliana* (Dormann et al., 2000, Pidkowich et al., 2007) and hairpin RNA-mediated post-transcriptional gene silencing of SAD was used in the generation of high-stearic, high-oleic (HS-HO) cottonseed oil (*Gossypium hirsutum*) (Liu et al., 2002).

Although *B. napus* engineered with increased saturated fatty acid content has not been a commercial success, market opportunities still exist for plant derived sources of 16:0 and 18:0, both for food and industrial uses. Temperate oilseed options for 18:0 include HS-HO sunflower

(*Helianthus annuus*), now entering commercial production (Dubinsky and Garcés 2011, Garcés et al., 2012) and perhaps HS soybean (*Glycine max*) (Clemente and Cahoon 2009), both produced by conventional breeding. Demand for 16:0 is largely met by palm oil (approximately 44% 16:0), or cottonseed oil (approximately 26% 16:0).

Our objective is to further examine the potential of *B.napus* as a platform for saturated fatty acid production, particularly for the temperate production of 16:0 to provide an alternative to palm oil. As the first step in our research, we achieved the simultaneous over-expression of a cDNA encoding a native FatB thioesterase and down regulation of *SAD* gene expression, generating plants containing up to 46% saturated fatty acids. For precise silencing of specific genes encoding SAD we used an artificial micro-RNA mediated approach (amiRNA). The creation and analysis of these engineered *B. napus* plants is described.

Results

Isolation of *Brassica napus* cDNAs encoding FatB and SAD isoforms

To identify a *B.napus* gene encoding a palmitoyl-ACP-thioesterase (FatB) for over-expression, the GenBank database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) was searched for known *FatB* genes from this species. A single sequence was identified, accession number DQ847275, and both the nucleotide sequence and deduced amino acid sequence (accession ABH11710) were used to search the *B.napus* ESTs database (http://brassicagenomics.ca/ests/blast.html). Assembly of identified ESTs indicated that, in addition to a sequence corresponding to DQ847275, a second transcript encoding a member of the *FatB* gene family in *B. napus* was also represented. A full length cDNA representing the second *FatB* gene (designated *BnFatB(2)* in this work) was then amplified by RT-PCR using mRNA isolated from developing seeds of *B. napus* cv. DH12075.

The *BnFatB(2)* open reading frame (ORF) was 1,248 bp in length, encoding a deduced precursor protein with a length of 415 amino acids (GenBank Accession KC202816). The coding regions of the two presently known *B. napus FatB* cDNAs, *BnFatB(2)* and DQ847275 are highly conserved, with 88 % homology at the nucleotide level and 89 % identity at the amino acid level. The deduced precursor proteins (compared in Fig.1) encoded by *BnFATB(2)* and DQ847275 have molecular masses of 46.2 kDa and 46.0 kDa, respectively. Presence of a chloroplast transit peptide was predicted using ChloroP (Emanuelsson et al., 1999; http://www.cbs.dtu.dk/services/ChloroP/) with a predicted cleavage site between amino acid

residues Q91 and L92 in BnFATB(2), based on the structure of the mature Arabidopsis FatB (Mayer et al 2007), resulting in a predicted mature polypeptide of 324 aa. The deduced mature proteins coded by *BnFATB(2)* and DQ847275 have molecular masses of both 36.7 kDa, and isoelectric point of 6.37 and 6.86, respectively. The three amino acid residues of the catalytic triad (N,H,C), essential for catalytic activity of FatB thioesterases, belonging to the conserved active-site motifs NQHVNN and YRRECG (Yuan et al., 1996, Mayer et al., 2007), were found in BnFatB(2) mature polypeptide as N318, H320 and C355, respectively. Six amino acid residues (V164, K177, V201, M232, S265 and W312) related to the substrate specificity (Mayer et al., 2007) are also conserved. The novel *BnFatB(2)* cDNA was selected for over-expression in *B. napus*.

A similar strategy was used to identify ESTs encoding putative stearoyl-ACP desaturase homologues. The *B. napus* EST database were searched using previously identified *SAD* cDNAs, accession X74782, X97325, X63364 and AY642537. By sequence comparison (Table S1 and Fig. S1) it was determined that accessions X63364 and AY642537 likely represent the same gene from two different *B. napus* cultivars. Assembly of overlapping ESTs identified a total of 8 sequences predicted to encode full length *SAD* homologues or orthologues. Assembled sequences were Bn2745, Bn8595, Bn25810, Bn240, Bn47973, Bn47975, Bn47974 and Bn47976 and available as supplemental data. Four sequences had high sequence homology to Arabidopsis *FAB2/SSI2* (At2g43710), encoding the chloroplast stearoyl-ACP desaturase. The remaining 4 sequences showed highest deduced amino acid similarity to Arabidopsis DES5 (At3g02630) and were designated BnDES5a to BnDES5d (Table S2). As *B. napus* is an allotetraploid (AACC) we determined the likely genome of origin for the 4 FAB2/SSI2 homologues by comparison of cDNA and deduced amino acid sequences with available sequences from the diploid species *B. oleracea* (CC) and *B. rapa* (AA) using the NCBI GSS (Genome Survey Sequence) database (http://www.ncbi.nlm.nih.gov/projects/dbGSS/), and more recently the *B. rapa* genome sequence assembly using the *Brassica* database (BRAD, http://brassicadb.org/brad/index.php, Cheng et al., 2011). Based on this analysis, sequences were designated *BnaA-SAD1*, *BnaA-SAD2*, *BnaC-SAD1* and *BnaC-SAD2* as shown in Table S2. A cladogram illustrating the relationship between the BnSAD/DES sequences and the Arabidopsis stearoyl-ACP desaturase family is given in Fig. 2. To determine the accuracy of our assembly of the EST sequences, a 1182bp cDNA with 100% sequence identity to sequence *BnDES5b* was amplified by RT-PCR using mRNA isolated from developing seeds of *B. napus* cv. DH12075 (KF256138). The deduced amino acid sequence (Fig. S2) of 393 residues had

94.4% identity to Arabidopsis Des5 (At3g02630), a predicted precursor-protein molecular mass of 44.8 kDa, and a putative N-terminal chloroplast transit peptide. A consensus binding motif [(D/E)X2H]2 characteristic of di-iron proteins (Shanklin and Cahoon 1998) was also observed.

Creation and verification of transgenic *B. napus* lines with down-regulated *SAD* expression and up-regulated FatB activity.

To simultaneously over-express a *FatB* thioesterase cDNA and down-regulate the four *B. napus* homologues of Arabidopsis *FAB2/SSI2* and the four *AtDES5* like genes, a binary vector was constructed. This contained the *FATB(2)* cDNA under control of a napin promoter for seed specific expression, accompanied by two amiRNAi cassettes designed to target all 8 *SAD* family genes, also under control of a napin promoter. Design details are given in the experimental procedures section. After *Agrobacterium* mediated plant transformation a total of 52 transformants were recovered. All were screened by PCR and the presence of the intact transformation cassette was confirmed in 33 individuals. Eight plants showed no cassette integration and were likely escapes. Initial characterization was conducted by GC-FAMES analysis of seed fatty acid composition (Fig. 3). The majority of the plants exhibited a major increase in total saturated fatty acid content ranging from 7.4% in the control DH12075 line to 43.5% in line transgenic line 48, with palmitic acid being the predominant saturated fatty acid component. Lines with no detectable transgene cassette (plants 3,9,14,27,30) had saturated fatty acid composition similar to the untransformed control. Transgene locus number for each line was determined by germinating T1 seeds in the presence of kanamycin and three lines (lines 39, 43 and 48) were selected for further analysis based on transgene locus number, and the saturated fatty acid content of the T1 seeds. Kanamycin screening indicated that plant 39 was a single insertion line, plant 43 contained the transgene cassette in two loci, and plant 48 most likely contained the transgene cassette in 3 loci. Segregation for kanamycin sensitivity/resistance in the T1 seeds was 17/55, 5/85 and 2/118 for the three plants respectively. Transgene copy number was also confirmed by the segregation ratio of 16:0 in single T1 seed, by GC analysis of FAMES. T1 seed was planted and 6 homozygous plants (Bn39-17, Bn39-40, Bn39-46, Bn43-5, Bn48-93 and Bn48-115) were selected for detailed characterization. All 6 contained the intact transformation cassette, as evidenced by PCR with genomic DNA as template (Fig. S3).

To demonstrate that the artificial microRNA was correctly expressed in the developing seed we analyzed the expression of the two amiRNAs by stem-loop RT-PCR in both 21 and 28

DPA developing seeds. The results from all six transgenic *B. napus* lines showed similar expression levels at 21 DPA and 28 DPA. Both BnSADamiR1 and BnSADamiR2 were highly expressed in 21 DPA developing seeds in the transgenic lines with no expression detected in the developing seeds of untransformed DH12075. Data from stem-loop RT-PCR analysis in 21 DPA developing seeds is shown in Figure S4.

To monitor changes in the expression levels of the target genes we chose an RT-PCR based approach, using a *B.napus* actin transcript as an internal control. Initially, the relative expression levels of *BnFATB*s, *BnSAD*s and *BnACTIN* in 21 DPA developing seeds of untransformed *B.napus* DH12075 was analyzed by RT-PCR using gene specific primers and the same number of amplification cycles for all primer pairs. These results indicated that *BnaA-SAD1* and *BnaA-SAD2* (Fig. S5, lanes 4 and 5) have the highest expression levels among the genes investigated, and the two *BnFatB* genes (Fig. S5, lanes 2 and 3) were expressed at similar levels. Differences in amplification product size with each primer pair ensured that specific gene products could be efficiently distinguished. The semi-quantitative RT-PCR was then repeated for the control line and the 6 transgenic lines (Fig. 4). Results demonstrated that the transcription level of the over-expressed *BnFatB2* gene in all the six transgenic lines was more than 10 fold higher compared to the endogenous expression seen in the untransformed DH12075, indicating successful over-expression. Expression of the endogenous *BnFatB* gene corresponding to the DQ847275 sequence, as determined by gene specific primers, appeared unchanged in the transgenic lines. The expression levels of all 4 *BnSAD*s and *BnDES5*a-d were all reduced in the six transgenic lines, but transcript of all genes was still detected. The three plants from line 39 showed the strongest down regulation of *BnaA-SAD2*.

Determination of fatty acid composition and seed oil content

The fatty acid composition of leaves, young siliques, and mature seeds from *B. napus* control and transgenic lines was determined by GC-FAME analysis. To verify that the modified fatty acid composition was restricted to the seed, the average levels of (16:0 + 18:0) and total saturated fatty acids in 7-day-old leaves of all transformed T0 plants was determined and found to be 13.9 ± 1.5 % (±SD) and 23.2 ± 2.0 % (±SD), respectively. No obvious difference was observed compared to levels in DH12075 untransformed controls grown at the same time [12.8 ± 0.8 % (±SD) and 26.0 ± 0. 7 % (±SD), respectively]. Levels of (16:0 + 18:0) and total saturated fatty acids in 5 PDA siliques of the T0 plants were 20.8 ± 1.5 % (±SD) and 31.3 ± 3.6 % (±SD), respectively, with no obvious difference compared to that of DH12075 [21.9 ± 0.2 % (±SD) and

28.2 ± 1.5 % (±SD)]. The transformed lines clearly did not differ significantly from control plants in the fatty acid composition of leaf or young siliques, as would be expected with transgenes placed under control of the seed specific napin promoter.

Seed lipid composition of T2 seeds from the 6 transgenic lines Bn39-17, Bn39-40, Bn39-46, Bn43-5, Bn48-93 and Bn48-115, on which detailed molecular characterization had been conducted, is given in Table 1. Total saturated fatty acid content was increased from approximately 7.4% in the control line to 45.6% in Bn48-93, with all lines maintaining the high saturated fatty acid profile seen in the screening of pooled T1 seed. Increase in saturated fatty acid content was largely accounted for by an increase in 16:0, with smaller relative increases seen in 18:0, 20:0 and 22:0. In line Bn39-17 for example, 16:0 increased over 7 fold compared to the control line, with a 3 fold increase in 18:0 and 20:0. The medium chain fatty acid myristic acid (14:0) was observed in all lines with increased saturated fatty acid content, but was not a significant component of the seed oil of the untransformed control. Levels of all monounsaturated fatty acids were reduced, with the greatest reduction seen in oleic acid ($18:1^{\Delta 9}$). In contrast the percentage of linoleic acid ($18:2^{\Delta 9,12}$) was slightly increased in all lines except Bn48-93. Percentage of linolenic acid ($18:3^{\Delta 9,12,15}$) showed considerable variation between the lines, with those derived from the T0 plant BN39 not differing significantly from the control, whereas the other lines showed a small reduction. The minor *n-7* fatty acids $16:1^{\Delta 9}$ and $18:1^{\Delta 11}$ were both reduced in the transgenic lines.

To examine whether saturated fatty acid content was increased in seed polar lipids, total lipid was extracted from mature seeds of DH12075 plants, and two transgenic lines (T3 seeds, plants Bn39-17-1 and Bn48-115-1) and separated into TAG and polar lipid fractions by TLC for analysis of fatty acid composition (Fig. 5). In the DH12075 seeds, 16:0 accounted for 9.7% of total fatty acids in the polar lipid fraction. Levels increased approximately 2 fold to 20% in the transgenic lines. In contrast, the levels of 16:0 in TAG increased approximately 7 fold from 4.4% in the DH12075 seeds to 27.5 and 28.7% in the Bn39-17-1 and Bn48-115-1 seeds respectively. The increase in 16:0 in the polar lipids and TAG of the transformed lines was accompanied by a significant decrease in 18:1, with a small increase in 18:2 and a slight decrease in 18:3.

Seed oil content was determined from pooled T2 seeds from the 6 transgenic lines, and from control plants grown at the same time. Average oil content of the control line was 41.8 ±1.4% (±SD) with an oil content of 48.3 ±0.4 % (±SD) recorded for line Bn43-5 (Fig. 6A), as measured by NMR. Although line BN43-5 had considerably higher oil content compared to the control,

lines Bn39-40 and Bn48-115 had slightly reduced oil content and no clear pattern was obvious from these plants. We therefore returned to the T1 seeds for all of the transgenic lines and compared average oil content to 16:0 saturated fatty acid composition over the entire population (Fig. 6B) Analysis of the results indicated that a weak correlation (R^2 =0.05) was seen with increased 16:0 content appearing to correspond to a slight decrease in seed oil content. Taking data from the analysis of T2 seeds into consideration, is seems that the increase in saturated fatty acid does not have a major impact on oil content.

Analysis of triacylglycerol by MALDI-TOF MS and physical properties of the seed oil.

As the transgenic lines exhibited dramatically altered fatty acid profiles, we examined the seed triacylglycerols by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Spectra were collected between 800 *m/z* and 1000 *m/z* for oil samples from line Bn48-115 and the control DH12075 line. Results (Fig.7) showed a shift from predominantly C54 TAG species (where the number indicates the total number of carbons in the fatty acid components) in DH12075 such as tri-oleate OOO (*m/z* = 907.8), OOL (*m/z* = 905.8), OOLn (*m/z* = 903.8), enriched in oleic acid (O), linoleic acid (L) and linolenic acid (Ln), to predominantly C50 and C52 TAGs in Bn48-115 enriched in palmitic acid (P) and stearic acid (S), such as POP (*m/z* = 855.8), PLP (*m/z* = 853.7), PLnP (*m/z* = 851.7), POO (*m/z* = 881.8), POL (*m/z* = 879.8), PLL (*m/z* = 877.7), POS (*m/z* = 883.8), PLS (*m/z* = 881.8), PLnS (*m/z* = 879.8). The three most intense ions identified in the Bn48-115 TAG region, confirmed by MALDI-TOF MS/MS (data not shown) were attributed to POO (*m/z* 881.8), POL (*m/z* = 879.8) and PLP (*m/z* 853.7) TAG species. For the control oil sample DH12075 the three dominant ions identified in the TAG region were identified as OOO (*m/z* = 907.8), OOL (*m/z* = 905.8) and OOLn (*m/z* = 903.8). The increase in saturated fatty acid content of the seed oil clearly corresponded to a major increase in TAG species containing a single palmitate moiety, and the appearance of substantial amounts of TAG containing two molecules of palmitate, TAG species that were largely absent in oil from the control seeds.

To assess the physical properties of the oil, melting point was determined for hexane extracted oil prepared from seeds of the control plants and the 6 transgenic lines. Results (Table 1) indicated that the melting point temperature of the oil from transgenic plants was greatly increased at 12.0 °C for Bn48-115 and 15.0 °C in Bn48-93, more than 20 °C higher than the melting temperature of -10 °C observed for oil from the control DH12075 seeds. Oil from the different transgenic lines chilled to 6 °C for 20 minutes is shown in Fig. 8. All samples from the

transgenic plants were solid at this temperature suggesting a potential use as a spreadable fat with zero trans-fatty acid content.

Seed germination and seedling growth at low temperature

When conducted at room temperature (22°C), seed germination and seedling establishment for all 6 transgenic lines was comparable to the control DH12075, irrespective of the saturated fatty acid content of the seed oil. Plants grew normally under greenhouse conditions (Fig 9A). When the seeds were germinated at low temperature, inhibition of seedling establishment was observed. As shown in Fig. 9B, seeds of the six lines were able to germinate when incubated at 6°C for 13 days, but root extension was severely inhibited in most lines and greening of cotyledons was reduced. The most severely affected line was Bn48-93, the line with the highest saturated fatty acid content (45.6%).

Discussion

The objective of this work was to produce a prototype high saturate oil in a *B. napus* canola cultivar. This oil could provide an alternative to palm oil as a source of palmitic acid, or be used for blending or interesterification to produce margarine or shortening. The use of canola, with around 23% polyunsaturated fatty acid (PUFA) content, would distinguish the oil from cotton seed oil, a source of palmitic acid in a high PUFA background. The approach was to use a combination of *FatB* over-expression to enhance palmitate release from ACP while simultaneously down-regulating *SAD* expression to block desaturation of stearoyl-ACP. Based on previous work suggesting that FatB activity may in part be influenced by ACP structure (Salas and Ohlrogge, 2002), we chose to use a native *FatB* for the over-expression work and therefore cloned a cDNA encoding a novel *FatB* from *B. napus*. To down-regulate *SAD* expression by the highly gene specific method of artificial microRNA mediated gene silencing (Warthmann et al., 2008) it was first necessary to identify all target genes in *B. napus*. Due to the nature of *Brassica napus*, an allotetrapoid (2n=4×=38, AACC) thought to have originated through spontaneous interspecific hybridization between *Brassica rapa* (2n=20, AA) and *Brassica oleracea* (2n=18 CC (U, 1935, Parkin et al., 1995), SAD activity was expected to be encoded by multiple genes. As no genome sequence was available for any *Brassica* species when the work described here was initiated, we searched all available *B. napus* EST resources

using Arabidopsis sequences as reference and identified 4 genes encoding proteins with high sequence identity to Arabidopsis *FAB2/SSI2* (At2g43710) and 4 with high amino acid sequence identity to Arabidopsis *DES5* (At3g02630, Kachroo et al., 2007). By RT-PCR (Fig.S5), all 8 genes were shown to be expressed in developing *B. napus* seeds. Previous work has shown that *B. napus* contains 4 genes encoding stearoyl-ACP desaturase (Slocombe et al., 1994), cDNAs representing three of these genes were available in public databases and we were able to clone a 4th. The Arabidopsis stearoyl-ACP desaturase gene family, however, contains seven members (Kachroo et al., 2007). These are present as a tandem triplet on chromosome 3 (At3g02610, At3g02620 and At3g02630, designated *DES2*, *DES4* and *DES5* respectively), a tandem pair on chromosome 5 (At5g16230 and At5g16240, designated *DES3* and *DES1*) and 2 single genes on chromosomes 1 and 2 (At1g43800 and At2g43710, *DES6* and *FAB2/SSI2*). The *FAB2/SSI2* locus (At2g43710) encodes the SAD largely responsible for the desaturation of 18:0 to 18:1 in plastids throughout the plant (Lightner et al., 1994). Although the biological role of DES5 in Arabidopsis is currently unknown, the enzyme has been shown to catalyze the $\Delta 9$ desaturation of 18:0-ACP (Kachroo et al., 2007). To ensure the highest probability of down-regulating SAD activity, our strategy was to target all 8 genes identified as being expressed in developing *B. napus* seed, not just the four *FAB2/SSI2* homologues.

With the recent release of the *B. rapa* (Chiifu-401) genome sequence (Wang et al., 2011) complemented by syntenic gene analysis (Cheng et al., 2012), it is now possible to examine the SAD gene family in the diploid species representing the A genome of *B. napus*. Like Arabidopsis, the *B. rapa SAD* gene family also contains 7 genes. As illustrated in Fig. 2, the *B. rapa* genes Bra000321 and Bra008631, located on chromosomes A03 and A05 respectively, are most likely orthologues of Arabidopsis *FAB2/SSI2*. Bra008631 and Bra008632 located in tandem on chromosome A10 appear to be orthologues of the Arabidopsis tandem pair *DES1* (At5g16240) and *DES3* (At5g16230). No orthologues of Arabidopsis *DES6* (At1g43800) were observed. By sequence comparison, the *B. rapa* genes Bra021427 and Bra001057 are orthologues of Arabidopsis *DES5* whereas Bra039178 encodes a protein with high amino acid identity to both DES2 and DES4. Examination of the genomic sequence adjacent of the predicted Bra039178 ORF, using FGENESH+ (http://linux1.softberry.com/) uncovered an additional initiation codon 42bp upstream, giving a predicted amino acid sequence of 409 residues for the encoded protein. Both potential translations result in a protein with higher amino acid identity to Arabidopsis *DES2* (90%) than *DES4* (85%). A previous detailed study of SAD loci in the *B. napus* genome and the dipoid A and C genomes, using BAC sequencing (Cho et

al., 2010) presented evidence that the regions in those genomes corresponding to the Arabidopsis tandem triplicate of *SAD* genes At3g02610, At3g02620 and At3g02630 (*DES2*, *DES4* and *DES5* respectively) contains only a single gene. The conclusion was that the *Brassica* genomes contained no genes equivalent to At3g02610 and At3g02620 (*DES2* and *DES4*) and a single gene representing the At3g02630 (*DES5*) orthologue. The published *B. rapa* genome sequence indicates that there are 2 *DES5* orthologues on chromosomes A01 and A03, and a single *DES2* orthologue on chromosome A05 (Figure S6A), suggesting a complex rearrangement. No gene orthologous to of Arabidopsis *DES4* (At3g02620) was observed in the *B. rapa* genome sequence assembly and this gene is also absent from *Arabidopsis lyrata* (Fig. S6B). The presence of 2 orthologues of Arabidopsis *FAB2/SSI2* and 2 orthologues of *DES5* in *B. rapa* supports our assembly of ESTs representing 4 genes for *FAB2/SSI2* and 4 for *DES5* in *B. napus*. Further clarification is expected on release of the *B. oleracea* and *B.napus* genomes.

The transformed *B. napus* plants showed a major increase in saturated fatty acid content with 16:0 as the predominant fatty acid, suggesting that the phenotype observed was largely due to the activity of the over-expressed BnFatB2. The anticipated phenotype of high 16:0 in combination with high 18:0 levels was not observed. The moderate increase in 18:0 and its elongation products 20:0 and 22:0 suggests a slight decrease in SAD activity, although FatB activity may also contribute to this increase. We did not conduct over-expression of the *BnFatB(2)* cDNA alone in *B. napus*, however ectopic expression of *BnFATB2* in Arabidopsis resulted in increased levels of 16:0 in the seed oil accompanied by a small increase in 18:0 (results not shown). As discussed previously, with the exception of the *FatB* from nutmeg (*Myristica fragrans*), only small increases in 18:0 were observed on *FatB* over-expression in *B. napus* (Jones et al., 1995, Hawkins and Kridl, 1998, Voelker et al., 1997) or Arabidopsis (Dörmann et al., 2000). Further evidence of efficient *SAD* down-regulation is a reduction in oil content, accompanied by a characteristic increase in 18:3 and VLCFAs levels, resulting from changes in flux through the fatty acid desaturation and elongation pathways (Knutson et al., 1992, Cernac and Benning 2004). Significantly increased 18:3 was not seen in the transformed lines, but variation in 18:2 and 18:3 content between the lines may reflect alterations in flux between the pathways of fatty acid modification and TAG assembly.

Molecular analysis clearly demonstrated the presence of the silencing amiRNA and a significant reduction in expression of all 8 target *SAD/DES5* genes. We examined amiRNA expression at a development time when seeds were actively synthesizing oil (Fig. 4, 21 DPA), and it is clear that the transgenes were expressed at an appropriate time to influence oil

composition. Previous modification of *B. napus* by antisense targeting of the *SAD* genes has successfully raised stearate levels to 40% of total seed fatty acids (Knutzon et al., 1992 Zarhloul et al., 2006). Although the previous studies did not report transcript levels, analysis of developing seeds by western blot demonstrated an almost complete loss of SAD protein (Knutzon et al 1992) suggesting very efficient gene down-regulation. *SAD* transcript in *B. napus* is highly expressed in the developing seed (Slocombe et al., 1992 and Fig.S5) and our results suggest that the amiRNA mediated silencing approach may not have achieved sufficient silencing to significantly reduce SAD activity. Transcript encoding SAD in *B. napus* seeds appears to be present in considerable excess of the level required for efficient desaturation of 18:0.

In Arabidopsis it is clear that although the SAD homologue FAB2/SSI2 is the primary enzyme responsible for 18:0 desaturation, other enzymes also contribute to 18:1 biosynthesis. The Arabidopsis *FAB2/SSI2* gene was first identified in an EMS-induced mutant population (James and Dooner 1990). Although sequencing of the *FAD2/SSI2* gene from this mutant identified a point mutation resulting in a truncated, and likely inactive, protein (Kachroo et al., 2001) the mutant still accumulates significant amounts of C18 unsaturated fatty acids and 20:1 in the seed oil. Subsequent identification and characterization of the additional 6 genes of the Arabidopsis *SAD/DES* gene family indicates that DES1 and DES5 catalyze the Δ9 desaturation of 18:0-ACP whereas DES3 preferentially catalyzed 16:0-ACP desaturation, all with very much lower *in-vitro* activity than FAB2/SSI2. The native expression of these genes was not able to compensate for the *fab2/ssi2* loss of function mutation and T-DNA insertions in *DES1* and *DES4* did not alter 18:0 levels in any of the tissues analysed (Kachroo et al., 2007). Without further knowledge of the extent of the *B. napus SAD/DES* gene family, and the activities of their encoded proteins, the contribution to 18:0 desaturation of enzymes other than the *SAD* and *DES5* homologues targeted here is unclear. A repeat of this work using an alternative silencing technique, or with the very effective antisense technique reported earlier (Knutzon et al., 1992), may offer new insights into the results observed here. Artificial microRNA mediated methods have been successfully applied to silence genes of lipid biosynthesis in Arabidopsis (Belide et al., 2012) but there is no published systematic study of their effectiveness in *B. napus*. From our analysis, we were not able to determine any biological role for the *B. napus* DES5 gene products.

Unlike pure compounds, natural vegetable oils do not have true melting points as they are made up of complex mixtures of TAG molecules that pass through a gradual softening

before becoming completely liquid (O'Brien, 1998). This is further complicated by the fact that oil crystals can exist in several polymorphic states (Fasina et al., 2008). The melting point, as determined by the oil becoming completely liquid in phase, for the oils from the transformed plants ranged from 7°C to 15°C, compared to -10°C for the control. This feature suggests that the oils may have some potential for direct use. From a nutritional aspect, low saturated fatty acid intake is currently considered desirable, however the metabolic effects of saturated fatty acids differ according to chain length (German and Dillard 2004) and many recent studies are now questioning the association between dietary saturated fatty acids and cardiovascular disease (Lawrence 2013). Furthermore, the structure of plant derived 16:0 rich TAG may have some health benefits compared to oils where saturated fatty acids are introduced randomly by hydrogenation or interesterification. In most vegetable oils, saturated fatty acids are located in *sn*-1 and *sn*-3 positions of triacylglycerols, polyunsaturated fatty acids (specifically linoleic and linolenic acids) occupy *sn*-2 position (middle), and monoenoic acids are relatively evenly distributed in *sn*-1, *sn*-2 and *sn*-3 positions; longer-chain fatty acids (C20-C24) are apparently concentrated in the primary positions with some small preference for position *sn*-3; less-common fatty acids tend to be concentrated in position *sn*-3 (Christie, et al., 1991). Analysis of the oils by MALDI-TOF MS demonstrated that 16:0 was distributed throughout the TAG, with the predominant species being C52 TAGs (C16+C18+C18) and a significant amount of C50 TAG (C16+C18+C16). In humans, digestion of TAG by endogenous lipases generates free fatty acids and *sn*-2-monoacylglycerol (*sn*-2-MAG) (Mu and Hoy 2004). Exclusion of saturates from the *sn*-2 position of TAG would however mean that digestion of the oil would generate *sn*-2 MAGs that were largely devoid of saturated fatty acids. The relatively poor intestinal absorption of 16:0 free fatty acid would further limit saturate uptake.

Of some concern, and a trait that requires further characterization, is the poor seedling establishment observed when seedlings were grown at low temperature. Oilseeds germination and seedling establishment is correlated with the degradation of storage lipids catabolized by the action of lipases (Quettier and Eastmond, 2009, Theodoulou and Eastmond, 2012). Germinating seeds generally express multiple lipases and no discrimination against saturated fatty acids has been reported, with most lipases now considered to be promiscuous (Kapoor and Gutpa, 2012). Studies with sunflower (*Helianthus annuus*) seed lipases did not show a marked preference for any TAG species and TAG degradation in high and low saturated fatty acid varieties proceeded at a similar rate during germination (Fernández-Moya, et al., 2000). Studies carried out with seeds of *Pinus edulis* indicated that the lipases were not specific for

individual TAG during germination, and levels of all TAG were depleted similarly (Hammer et al., 1994). Earlier work (Lin et al., 1986), however, suggested a correlation between lipase specificity and the fatty acid composition in the seeds of plants making unusual fatty acids including castor bean (*Ricinus communis*, ricinoleic acid), rapeseed (erucic acid), and elm (*Ulmus americana*, medium chain fatty acids). Germination and oil breakdown in the *B. napus* high 16:0 lines would likely result in enrichment of the seedling acyl-CoA pool with saturated fatty acids. Incorporation of these fatty acids into membrane lipids during seedling establishment may play a role in poor establishment at low temperature. Our analysis of seed polar lipids indicated that they already contained more than twice the percentage of saturated fatty acids found in the control line. The balance of saturated and unsaturated fatty acids in membrane lipids is an important determinant of membrane fluidity adaption to low temperature (Nishida and Murata 1996). Further characterization to determine the reason for poor establishment and development of a solution to this problem is required if high saturated fatty acid canola is to become a viable crop. Although canola is a spring sown crop in Canada, night time temperatures may be low enough to impact seedling establishment.

Looking beyond the experimental prototype and potential agronomic concerns described here, it is envisioned that high palmitic canola could co-exist with other types if the crop was grown in an identity preserved system. Such a system is successfully established for high erucic rapeseed (*B. napus* HEAR) grown for industrial use. The future of high saturated fatty acid crops will necessarily be determined by market needs. The current low price of palm oil compared to other commodity vegetable oils suggests that demand for alternative sources of palmitic acid may be low. Co-engineering of these plants with enzymes that can modify palmitate to produce a higher value fatty acid, for example a monounsaturated fatty acid with a novel double bond position, may therefore be an alternative.

Experimental Procedures

Plant materials and growth condition

The *B. napus* cultivar used in this work was DH12075. Control and all transgenic lines were grown in pots with soil in a controlled greenhouse environment (16 h light/8 h dark, 24 °C/18 °C). Plants were bagged during bolting to ensure self-pollination, and flowers were tagged on opening (anthesis) to record the development stages of seeds.

Isolation of *BnFatB* and *BnDES5* cDNAs

The SV Total RNA Isolation System (Promega Corp. Madison, WI, USA) was used for RNA isolation from 0.2 g of developing seeds collected at 20 days post anthesis (DPA). SuperScript II Reverse Transcriptase (Invitrogen Inc., Burlington, ON, Canada) was used for first-strand cDNA synthesis. To amplify a full length open reading frame (ORF) encoding BnFatB(2), primer pairs BnEstFatbFullUp (5'-ATGGTGGCCACCTCTGCTACATCCTC) and BnEstFatbFullDo (5'-TTACGATGTAGTGTCCCAAGTCG) were designed based on the sequences of two expressed sequence tags (ESTs), GenBank accessions EL587436 and FG567880. PCR was conducted using the PCR Platinum® kit with Pfx DNA Polymerase (Invitrogen) using the following conditions: 94 °C for 3 min., then 32 cycles of 94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 90 sec., with a final extension at 72 °C for 10 min. To amplify an ORF encoding BnDES5b, primer pairs BnEstSADFullUp (5'-AAAATGGCGATGGCTATGAG) and BnEstSADFullDo (5'-TTAAGCCCTAATCTCTCGATCA) were designed based on the sequences of three ESTs, EE536302, EE410520 and EE453671. TaKaRa Ex Taq™ DNA polymerase (EMD Millipore, Billerica, MA, USA) was used for amplification under the following conditions: 95°C for 3 min., then 35 cycles of 98 °C, 10 sec; 68 °C, 100 sec, with a final extension at 72 °C for 8 min. *B. napus* developing seeds cDNA was used as template for both PCR amplifications.

Artificial microRNA design and transformation vector construction

The 8 sequences encoding *BnaA-SAD1*, *BnaA-SAD2*, *BnaC-SAD1*, *BnaC-SAD2* and *BnDES5a* to *BnDES5d* were used for amiRNA design. Using website (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) two amiRNA fragments were designed, BnSADamiR1 (target: *BnDES5a, BnDES5b, BnDES5c and BnDES5d*) and BnSADamiR2 (target: *BnaA-SAD1, BnaA-SAD2, BnaC-SAD1* and *BnaC-SAD2*). AmiRNAs are designed to resemble natural miRNAs using three criteria: they start with a U, they display 5' instability relative to their amiRNA*, and their 10th nucleotide is either an A or an U. The strand with lower thermodynamic stability at its 5' end (5' instability) is preferentially incorporated into RISC. In designing amiRNA the following rules are also applied: no mismatch between positions 2 and 12 of the amiRNA for all targets; one (or two) mismatches at the amiRNA 3' end (pos.18-21); similar mismatch pattern for all intended targets; absolute hybridization energy between -35 and -38 kcal/mole (http://wmd.weigelworld.org/). The primers for engineering amiRNAs and site-directed mutagenesis on precursors of endogenous miRNAs were designed by the artificial microRNA designer WMD (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Primer designing

for *BnSAD/DES* amiRNAs was based on RS300 sequence (miR319a *A. thaliana*) gifted by Prof. Detlef Weigel, Max Planck Institute for Developmental Biology, Germany. The cloning strategy and protocol for engineering artificial microRNAs using 4 oligonucleotide sequences (I to IV) and RS300 A and B into the endogenous miR319a precursor is available at the following website (http://wmd2.weigelworld.org/themes/amiRNA/pics/Cloning_of_artificial_microRNAs.pdf). Fig. S7 illustrates the procedure used to generate the amiRNAs. The sequence of oligonucleotides I to IV for BnSADamiR1 and BnSADamiR2 is given in Table S3. Sequences of BnSADamiRNAs and their targeted regions for *BnSAD* and *BnDES5* genes in *B. napus* are given in Table S4.

The two individually engineered BnSADamiR1 and BnSADamiR2 fragments in the miR319a precursor from pRS300 were fused with a napin promoter and a nos terminator (nos-T) to form two expression cassettes, Pnapin-BnSADamiR1-NOS-T and Pnapin-BnSADamiR2-NOS-T by over-lapping PCR. The *BnFATB(2)* ORF was inserted between the napin promoter and a nos-T terminator to form a third expression cassette, Pnapin-BnFATB-NOS-T. All three expression cassettes were integrated into the binary vector pHS723 (Hirji et al., 1996) as shown in Fig. S7. The successfully constructed vector was named plasmid pLS571. In each cassette napin, a seed specific promoter, was used to ensure expression only in the developing seed.

Agrobacterium-mediated transformation of *B. napus*

Plasmid pLS571 was introduced into *Agrobacterium* strain GV3101: pMP90 by electroporation. Colonies were picked from selection plates and grown overnight in LB medium with 50 mg/L each of kanamycin and gentamycin. Presence and integrity of the transformation vector was confirmed my miniprep and restriction enzyme digestion. *Agrobacterium* mediated transformation of *B. napus* cotyledonary petiole explants from 5-day-old seedlings was carried out essentially as described by Moloney et al., (1989) except the explants were inoculated by bulk immersion in Agrobacterium suspension and co-cultivation was carried out without plating the explants on medium as described in Lee (1996).

Transgene verification

Total genomic DNA was extracted from *B. napus* leaves using the following rapid DNA extraction method. Small pieces of fresh leaf was ground with a pestle in a sterile 1.5 ml microfuge tube containing 400 µl extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS). The ground material was vortexed and centrifuged at 13 000 rpm for 5 min. 300 µl of the supernatant was transferred into a microfuge tube with 300 ul isopropanol and

mixed by inverting gently. The tubes were incubated for 5 min at room temperature and then centrifuged at 13 000 rpm for 5 min. The pellet was washed with 70% ethanol, air-dried and resuspended in 50 µl of sterile water. Primer pairs (Table S5) designed to confirm the presence of the different components of the transgene cassette were used as described in the text. PCR was conducted using Platinum® Taq DNA Polymerase (Invitrogen) for all amplifications.

Gene copy number determination for engineered *B. napus* plants

Two methods were employed to determine the number of transgene loci and zygosity of the engineered plants. Screening for resistance to kanamycin, conferred by the NPTII marker gene, was conducted by soaking the seeds in 250 mg/L kanamycin solution at room temperature for about 24 hours under continuous illumination until radicle emergence was observed. Seeds were transferred to soil and kanamycin resistance was determined by scoring the emergence of green, undamaged cotyledons. In a second approach, seed fatty acid profiles for single seeds from the line of interest were determined by gas chromatography (GC) of fatty acid methyl esters (FAMES). Zygosity was estimated by calculating segregation ratios for 16:0 content.

Detection of BnSAD amiRNAs by stem-loop RT-PCR

To detect amiRNAs by step-loop PCR, amiRNA specific primers (Table S6) were designed using the protocol of Varkonyi-Gasic and co-workers (2007). Total RNA was isolated from 21 and 28 DPA developing seeds using the TRIzol® Reagent (Invitrogen Inc.). Using 1 µl of total RNA as template, cDNA synthesis was conducted with stem-loop RT primers (BnSADamiR1RT and BnSADamiR2RT) and SuperScript II reverse transcriptase, as described in the manufacturer's protocol (Invitrogen Inc.). The PCR step of stem-loop RT-PCR was carried out as illustrated in Figure S8, using one microlitre of cDNA as template in a 25 µl PCR reaction with Taq DNA Polymerase (Invitrogen). For detection of artificial microRNA BnSADamiR1 expression, PCR was performed with forward primer BnSADamiR1FW and reverse primer UniReverse, yielding PCR product BnSADamiR1PR with a size of 62 bp. For detection of artificial microRNA BnSADamiR2 expression, PRC was performed with forward primer BnSADamiR2FW and reverse primer UniReverse, giving PCR product BnSADamiR2PR with a size of 63 bp. PCR amplification conditions were: 3 min of initial denaturation at 94 °C, 40 cycles at 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, followed by a final extension at 72 °C for 10 min. Three % agarose gel was used for separation of the PCR products.

Detecting *BnFatB* and *BnSAD/BnDES* gene expression by semi-quantative RT-PCR

Total RNA was isolated from developing seeds at 21 and 28 DPA using the RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada). One microgram of total RNA was used as template for cDNA synthesis with oligo(dT)16 as reverse transcription primer and SuperScript II reverse transcriptase. RT-PCR was carried out using 1 µl of cDNA as template in a 25 µl PCR reaction, with Taq DNA Polymerase (Invitrogen) for all amplification. Specific primers for each target *BnFatB* and *BnSAD/BnDES* gene were designed with Primer 3 software (http://frodo.wi.mit.edu/primer3/) as given in Table S7. *B. napus* actin (BnACTIN) (Accession No. AF111812) was used as a control. PCR amplification conditions for *BnFatB*s, *BnSAD/BnDES* transcripts and *BnACTIN* were: 3 min of initial denaturation at 94 °C, 24 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, followed by a final extension at 72 °C for 10 min.

Determination of seed fatty acid composition

For determination of total seed fatty acid composition, single or groups of 12 pooled seeds (from the same plant) were crushed and placed in a Pyrex screw-cap tube with 2 ml of 1 M HCl in methanol (Supelco, Bellefonte, PA, USA) and 300 µL of hexane. The tubes were tightly capped and incubated at 80 °C overnight. After cooling, 2 ml of 0.9 % NaCl was added, and FAMEs were recovered by collecting the hexane phase, with dilution as necessary. Gas chromatography of FAMEs was conducted using an Agilent 6890N GC equipped with a DB-23 capillary column (0.25 mm x 30 m, 0.25 mM thickness; J & W; Folsom, Ca, USA) and flame ionization detector, as described previously (Kunst L, et al., 1992).

Seed oil content determination

Seed oil content was determined by using a Maran Ultra benchtop NMR instrument (Oxford Instruments Molecular Biotools Ltd., Oxfordshire, UK), following the manual's procedure. About 0.5g cleaned and weighted seeds from each plant were used for seed oil content analysis, with three replicates for each sample. Canola oil was used for calibration.

MALDI-TOF MS and MS/MS analysis

Two grams of seeds from transgenic lines Bn39-17, Bn39-40, Bn39-46, Bn43-5, Bn48-93, Bn48-115 and DH12075 were crushed in aluminum foil and transferred to a glass tube. Two mL of hexane was added and the tubes were capped tightly, vortexed intensely and centrifuged

to precipitate debris. The hexane was carefully transferred to a clean tube and evaporated under a steam of Nitrogen gas. Extracted oil samples for TAG analysis were dissolved in chloroform, and a modified pencil lead method (Black et al., 2006) was used for MALDI-MS analysis. Pencil lead was scribbled on the MALDI plate and sodium chloride solution was spotted on top to ensure that sodiated ions were the dominant ions in the mass spectra. Samples were analyzed on an AB 4800 Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (Applied Biosystems, LLC. Frederick, MD, USA) equipped with a diode-pumped 355 nm Nd:YAG laser, and a laser intensity between 3000 and 3500 was used for MS data collection. The ion extraction delay time was set to 1000 ns and positive ion, reflectron mode was used. All mass spectra were recorded as sums of 400 laser shots (800 ns) with *m/z* range from 500 to 2000.

Measurement of oil melting temperature

Twenty μL aliquots of the oil extracted for MALDI-TOF MS analysis were transferred to 200 μl glass GC vial inserts and frozen at -20 °C for 5 min to solidify the oil. The frozen tubes were transferred to a thermocycler set to 4°C and left for 20 min for temperature equilibration. Subsequently, the temperature was gradually increased, in 0.5 °C intervals, from 4 °C to 18 °C, with 5 min at each temperature. At each interval the tubes were quickly lifted from and returned to the thermocycler to observe oil phase and record melting temperature.

Fatty acid composition of TAG and polar lipids.

To determine fatty acid composition, lipids extracted as described for MALDI-TOF MS analysis were separated by TLC on aluminium backed silica gel G plates (Whatman Ltd., Maidstone, Kent, England) using hexane:diethylether:acetic acid (140/70/3) as solvent. Lipid areas were identified by iodine staining of strips cut from plate edges and centre. Silica gel containing lipids was scraped from the plate and directly transesterified using 1M HCl in methanol. For quantification, a lipid standard was added prior to transesterification. FAMES were determined by GC as described above.

Seed germination and seedling growth of transgenic lines at low temperature

To assess germination, harvested mature seeds were placed on plates with 1% Agar and home-made MS (Murashige and Skoog) medium (without sucrose) and/or in fresh water at different temperatures (4 °C, 6 °C and 10 °C) and kept for 5-15 days in a growth chamber, either

in darkness or with a 24 h photoperiod (300 µmol m^{-2} s^{-1} light intensity). To assess seedling establishment, seeds previously germinated at low temperature were allowed to continue to grow at the same temperature either in medium or in soil for a total of 13-27 days. Establishment was measured under a 24 h photoperiod (300 µmol m^{-2} s^{-1} light intensity). Germination rate, root length, hypocotyl length, plant fresh weight and leaf status were measured and recorded. Data was analyzed and calculated using Microsoft Excel software. All experiments were performed in three replicates.

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Table 1 Fatty acid composition of T3 seeds (Bn39-17, Bn39-40, Bn39-46 and Bn43-5) and T4 seeds (Bn48-93 and Bn48-115) from the six transgenic lines (±SD) and melting temperature of the extracted oil.

	% Fatty acid												
Lines	**14:0**	**16:0**	**16:1**	**18:0**	**18:1D9**	**18:1D11**	**18:2**	**18:3**	**20:0**	**20:1D11**	**22:0**	**Sat FA**	**Melting temp °C**
DH12075	ND	4.2 ± 0.1	0.2 ± 0.0	2.1 ± 0.2	64.8 ± 1.3	2.5 ± 0.2	12.8 ± 0.6	10.4 ± 0.8	0.7 ± 0.1	1.3 ± 0.0	0.4 ± 0.0	7.4 ± 0.0	-10.0
Bn39-17	1.3 ± 0,2	28.0 ± 2.0	0.1 ± 0,0	6.8 ± 0.5	33.7 ± 4.0	1.7 ± 0.1	14.8 ± 0.8	9.0 ± 1.6	2.1 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	39.0 ± 2.5	11.0
Bn39-40	1.4 ± 0.3	27.0 ± 0.9	0.1 ± 0.0	7.3 ± 0.4	30.5 ± 1.3	1.7 ± 0.1	15.0 ± 0.4	11.6 ± 0.4	2.3 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	38.9 ± 1.1	10.0
Bn39-46	1.2 ± 0.1	26.5 ± 0.8	0.1 ± 0.0	7.7 ± 0.4	36.6 ± 1.7	1.9 ± 0.2	13.5 ± 0.9	8.2 ± 0.8	2.1 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	38.3 1.1	8.5
Bn43-5	0.9 ± 0.1	24.0 ± 0.9	0.1 ± 0.0	8.9 ± 0.5	40.0 ± 1.6	1.9 ± 0.2	13.1 ± 0.7	5.8 ± 0.9	2.6 ± 0.3	0.9 ± 0.0	1.0 ± 0.1	37.3 ± 0.1	7.0
Bn48-93	1.3 ± 0.2	31.1 ± 2.0	ND	11.0 ± 2.4	28.8 ± 2.2	1.1 ± 0.2	10.9 ± 2.3	5.6 ± 2.7	2.2 ± 0.3	ND	ND	45.6 ± 1.5	15.0
Bn48-115	1.0 ± 0.2	26.8 ± 2.7	0.1 ± 0.0	7.8 ± 0.9	35.1 ± 4.3	2.1 ± 0.2	15.0 ± 1.3	7.5 ± 1.0	2.1 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	38.5 ± 3.0	12.0

ND: Non-detectable; mean ± standard deviation for n=3.

Figure legends

Figure 1

Deduced amino acid sequence comparison of *Arabidopsis thaliana* FatB (*At*FatB, AEE28300) and two FatBs from *B. napus*, (DQ847275 and BnFatB(2), KC202816). The predicted signal peptide (Mayer et al., 2007) is underlined. Conserved active site motifs (Yuan et al., 1996) are marked below with #, with the N, H and C residues of the catalytic triad marked in bold (#). Putative substrate determining residues identified by Mayer and coworkers (2007) and marked * with additional previously identified residues marked ^ (Yuan et al., 1996).

Figure 2

Cladogram with branch support values illustrating the relationship of the *Bn*SAD/DES deduced amino acid sequences, and the Arabidopsis stearoyl-ACP desaturase family (Kachroo et al., 2007).

Figure 3

Saturated fatty acids as percentage of total seed fatty acids determined from pooled seeds of primary transgenic lines (T1 seed). Lines marked with a red star (lines 39, 43 and 48) were chosen for further study. Numbers above the columns indicate number of transgene loci in the selected lines.

Figure 4

Semi-quantative RT-PCR analysis of the expression of *BnFatB*s and *BnSADs/BnDES5s*. Total RNA of each line was extracted from 21 DPA developing seeds. All amplifications were tested in a linear range, 24 cycles for *BnFatB*s, *BnSAD/BnDES5s* and *BnACTIN* (control). 1, DH12075; 2, Bn39-17; 3, Bn39-40; 4, Bn39-46; 5, Bn43-5; 6, Bn48-93; and 7, Bn48-115.

Figure 5

Fatty acid distribution in seed polar lipids and TAG of the control line DH12075 and representative transformed lines. Data are mean ± stdev for n=3.

Figure 6

Seed oil content determined for *B. napus* line DH12075 and 6 transformed lines (A). Measurements were made in triplicate by NMR. Correlation between percentage fatty acid and percentage 16:0 in a population of B. napus lines transformed with vector pLS571. Points represent data for individual transformed lines, measured from pooled T1 seeds. Data (A) are mean ± sdev for n=3 or n=9 (DH12075) and (B) mean ± stdev for n=3.

Figure 7

MALDI-TOF MS spectra (*m/z* 800-1000 region) of seed oil from *B. napus* line DH12075 (A) and transformed line Bn48-115 (B). Spectra were recorded in positive ion mode; the ions are sodium ion adducts. TAGs are labeled according to the total number of carbons in the fatty acid component.

Figure 8

Oils extracted from pooled seeds of different transgenic lines chilled to 6 °C. 1, DH12075; 2, Bn39-17; 3, Bn39-40; 4, Bn39-46; 5, Bn43-5; 6, Bn48-93; and 7, Bn48-115.

Figure 9

A. DH12075 and transgenic lines grown under greenhouse conditions. B. Seed germination and seedling establishment of transgenic *B. napus* lines at low temperature. Seeds germinated and grown at 6 °C for 13 days on A. T. medium.

Supporting information.

Additional supporting information may be found in the online version of this article;

Table S1. Sequence identities of *B.napus FAB2/SSI2* homologues.

Table S2. Accession numbers and nomenclature for sequences encoding putative *B.napus* acyl-ACP desaturases

Table S3. amiRNA and primer sequences for site-directed mutagenesis on precursors of endogenous miRNAs miR319a in pRS300.

Table S4 Sequences of BnSADamiRNAs and their targeted regions for *BnSAD* and *BnDES5* genes in *Brassica napus*.

Table S5. Primers for detecting different expression cassettes by PCR in *B. napus* transgenic lines.

Table S6. Artificial microRNAs and primer sequences.

Table S7. Primers for detecting BnFATBs, *BnSADs/BnDES5*s and *BnACTIN* expression by RT-PCR in *B. napus* transgenic lines.

Figure S1. Deduced amino acid sequence comparison of *B.napus FAB2* homologues.

Figure S2. Amino acid sequence comparison of AtDES5 and BnDES5b.

Figure S3. Verification of transgene cassette integrity by PCR amplification. For each reaction, lanes are: C1, pHS723 plasmid; C2, pLS571 plasmid; C3, DH12075 genomic DNA; 1, Bn39-17 genomic DNA; 2, Bn39-40 genomic DNA; 3, Bn39-46 genomic DNA; 4, Bn43-5 genomic DNA; 5, Bn48-93 genomic DNA; 6, Bn48-115 genomic DNA. PCR was conducted with primer pairs given below. Relationship between PCR products and the transgene cassette is illustrated below the panels. Panel A, primer pair PnapinFatBFor and PnapinFatBRev; PCR product A (1,354 bp). Panel B, primer pair FatBnosTPnapinFor and FatBnosTPnapinRev; PCR product B (789 bp). Panel C, primer pair FatBnosTPnapinFor and FatBnosTPnapinRev2; PCR product C (1848 bp). Panel D, primer pair FatBnosTPnapinFor and FatBnosTPnapinRev1; PCR product D (1697 bp). Primer sequences are given in Table S5.

Figure S4. Stem-loop RT-PCR analysis confirming the expression of both BnSADamiR1 and BnSADamiR2 in the 21 day-post anthesis developing seeds. 1, DH12075; 2, Bn39-17; 3, Bn39-40; 4, Bn39-46; 5, Bn43-5; 6, Bn48-93; and 7, Bn48-115.

Figure S5. Relative expression levels of BnFatBs, BnSAD/BnDES5s and BnACTIN in the developing seeds of B.napus determined by RT-PCR analysis. Total RNA of DH12075 (untransformed control) was extracted from 21 DPA developing seeds. PCR annealing temperature is 56 °C, totally 24 cycles. 1, *BnACTIN*; 2, *BnFatB, DQ847275*; 3, BnFatB(2); 4, *BnaA-SAD1*; 5, *BnaA-SAD2*; 6, *BnaC-SAD1*; 7, *BnaC-SAD2*; 8, *BnDES5a*; 9, *BnDES5c*; 10, *BnDES5b*; and 11, *BnDES5d*.

Figure S6. Genomic context of *DES5* orthologues. A. Diagram, not to scale, of orthologous genomic regions of *Arabidopsis thaliana* and *Brassica rapa* (Chiifu-401, chromosomes indicated). B. *Arabidopsis thaliana* and *Arabidopsis lyrata* (AI).

Figure S7. Construction of binary vector pLS571.

Figure S8. Illustration of stem-loop RT-PCR.

Contig sequence data.