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Hairpin-RNA mediated silencing of endogenous *FAD2* gene combined with heterologous expression of *Crambe abyssinica FAE* gene causes an increase in the level of erucic acid in transgenic *Brassica carinata* seeds

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Abstract The 3′-UTR of the *FAD2* gene from *Brassica carinata* was cloned by PCR and used to prepare an intron-spliced hairpin RNA (ihpRNA) construct. Compared to that of the wild type (WT) background, this construct, when expressed in *B. carinata*, resulted in a high degree of *FAD2* gene silencing accompanied by strong increases of up to 16 and 10% in oleic acid and erucic acid proportions, respectively. The increase in 18:1 was accompanied by a concomitant proportional reduction in 18:2. A second construct containing ihpRNA targeted to the endogenous *FAD2* gene in addition to the heterologous *Crambe abyssinica FAE* gene under the control of seed specific napin promoter, was used to transform *B. carinata*. This approach resulted in an even greater increase in erucic acid proportions, by up to 16% in T_1 segregating seeds as compared to that of the WT control. To our knowledge, this is currently the highest accumulation of erucic acid achieved in *B. carinata* seeds using transgenic approaches, making it an increasingly-attractive alternative to high erucic *B. napus* cultivars as an industrial oil crop.

Keywords Gene silencing · Hairpin RNA-mediated silencing (RNAi) · Oleate desaturase gene (*FAD2*) · Fatty acid elongase gene (*FAE*) · 3-Ketoacyl-CoA synthase gene (*KCS*) · Erucic acid · *Brassica carinata*

Database: The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank under accession number DQ250814.

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Introduction

The strategic goal of our research is to modify high erucic acid (HEA) germplasm of the *Brassicaceae* to increase the content of erucic acid (22:1Δ13) in the seed oil for industrial niche markets (Jadhav et al. 2005). HEA cultivars are of high interest for industrial purposes because 22:1 is a valuable feedstock with more than 1,000 potential or patented industrial

applications (Scarth and Tang 2006). Currently the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an anti-block or slip-promoting agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid, e.g. in lubricants, detergents, film processing agents and coatings, as well as in cosmetics and pharmaceuticals (Leonard 1993; Derksen et al. 1995; Basra and Randhawa 2002; McVetty and Scarth 2002; Puyaubert et al. 2005; Mietkiewska et al. 2007). For many of these industrial uses, the economics are limited by the proportion of 22:1 in the seed oil. With respect to the market for high erucic acid, it is estimated that about 80,000 tons of high erucic oil is used annually worldwide for lubricants, plastics, lacquers, and detergents (http://www.gov.mb.ca/agriculture/research/ardi/projects/98-022.html) Additionally, the European market for high erucic acid oil in 2005 was estimated at 55,000 MT with an annual growth rate of 4–5% (http://www.ienica.net/crops/crambe.pdf). A Brassica cultivar containing erucic acid at levels approaching 80% would significantly reduce the cost of producing erucic acid and its derivatives and could meet the forecasted demand for erucic acid as a renewable, environmentally friendly industrial feedstock (Leonard 1994; Taylor et al. 2002; Jadhav et al. 2005).

As we have shown earlier, over-expression of the *Crambe abyssinica FAE* gene in *Brassica carinata* resulted in a substantial increase in the proportion of erucic acid in seeds compared to the wild type control (Mietkiewska et al. 2007) (see schematic in Fig. 1). The synthesis of erucic acid in transgenic *B. carinata* plants was probably, in part, limited by the smaller microsomal pool of oleoyl-moieties (7–8%) available for elongation. As pointed out previously by Bao et al. (1998) and subsequently by Jadhav et al. (2005) the flux of 18:1 through distinct intermediate lipid pools before elongation might be a factor that limits the availability of 18:1 for elongation. The oleate desaturase, FAD2, is one of the crucial enzymes for the production of polyunsaturated fatty acids in plants (Okuley et al. 1994). As we have shown, by altering the level of *FAD2* gene expression using antisense and cosuppression approaches, it was possible to increase the pool of 18:1 available for elongation to enhance production of erucic acid in *B. carinata* seeds (Jadhav et al. 2005). However, the antisense and cosuppression strategies have variable and unpredictable effectiveness and require the production of large populations of transgenic plants to obtain a reasonable number of lines showing sufficient levels of target gene suppression (Liu et al. 2002).

The discovery that RNA interference in plants is mediated by sequence-specific degradation of dsRNA has led to the development of highly efficient methods of post transcriptional gene silencing (PTGS). Constructs specially designed to express dsRNA in plants in the form of self-complementary hairpin RNA (hpRNA) elicit a high degree and frequency of PTGS of endogenous genes (http://www.pi.csiro.au/RNAi/, Wesley et al. 2001; Stoutjesdijk et al. 2002). Such hpRNA constructs have great potential for genetic manipulation to improve crop traits (Wang et al. 2000).

We are advocating that *B. carinata* be developed as an alternative crop platform for industrial oil production and high-erucic oils in particular. *B. carinata* is easily transformed at very high efficiency (Babic et al. 1998), is highly disease (e.g. blackleg)-resistant, and is drought-resistant, amenable to growth in hotter, drier regions such as the brown soil areas of southern Saskatchewan. While *B. carinata* has outcrossing rate of 20–30% within its own species, a rate typical Brassicaceae (Murphy 2005), it fortunately also has a very low frequency of out-crossing to canola (Warwick and Black 1993), and therefore poses a lower risk of contaminating oils destined for the food chain. New breeding lines of *B. carinata* with higher oil and low glucosinolate content are currently being developed at Agriculture and Agri-Food Canada (Dr K. Falk, personal communication) and will provide excellent germplasm for production of high erucic and other industrial oils.

In the present study, we used a partial 3′-UTR of the seed-specific *B. carinata FAD2* gene to prepare an intron-spliced hpRNA construct to silence the seed *FAD2* gene and consequently, to increase the pool of oleic acid available for elongation. We also demonstrate how an increased pool of oleic acid can contribute to a dramatic increase in the content of erucic acid in Brassica seeds, particularly when combined with heterologous *C. abyssinica FAE* expression.

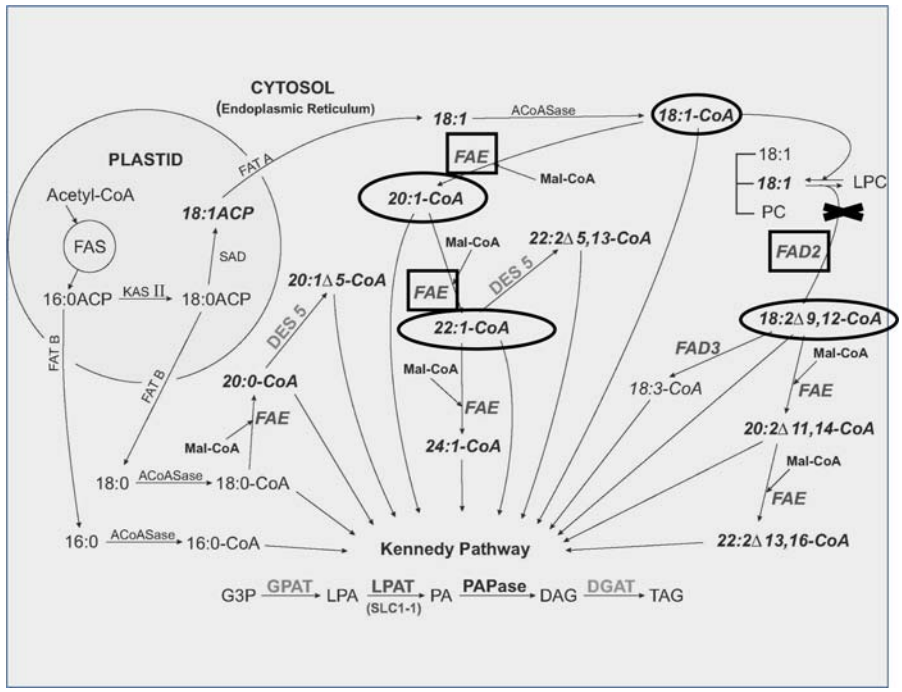

Fig. 1 Simplified schematic of fatty acyl metabolism and triacylglycerol (TAG) assembly in oilseeds, showing the salient ER-based reactions affected by the transformations performed in the present study. The enzymatic steps affected by the *Crambe FAE* and *B. carinata* RNAi *FAD2* are boxed. The affected pools of acyl-CoAs are circled. The reduction in flux from 18:1 to 18:2 due to the partial silencing of FAD2 which desaturates oleoyl moieties while on PC, is marked by an "X". The elongation of 18:1 to 20:1 and then 22:1 occurs via fatty acid elongase(s) (FAEs)

Materials and methods

Plant materials and growth conditions

Brassica carinata plants were grown under sterile conditions on MS medium (Murashige and Skoog 1962) during transformation and tissue culture. Transgenic *B. carinata* plants were grown in the greenhouse at the Kristjanson Biotechnology Complex greenhouses, Saskatoon, SK, under natural light conditions supplemented with high-pressure sodium lamps with a 16 h photoperiod (16 h of light and 8 h of darkness) at 22°C and a relative humidity of 25–30%.

Cloning of 3′-UTR of *FAD2* gene

The ORF sequence of seed-specific *FAD2*-gene (AF124360) from *B. carinata* was used to design the forward primer 5′-GTCTGCTACGGTCTCTA CCG-3′. 3′-RACE was performed using the SMART™ RACE kit (CLONTECH). A cDNA prepared from *B. carinata* developing seeds was used as a template for PCR amplification during 30 cycles of the following program: 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. A 686-bp PCR product was cloned into the pCR2.1-TOPO (Invitrogen) cloning vector and subsequently sequenced. Sequence comparison of the PCR product with the *FAD2* ORF sequence showed the presence of 236-bp 3′-UTR. The 3′-UTR sequence was submitted to GenBank (accession no: DQ250814).

Gene-silencing constructs

A 142 bp region of the *FAD2* 3′-UTR was amplified by PCR with primers: 5′-ctcgag GGATGATGATG GTTTAAGA-3′ (lower case shows restriction site for *Xho*I) and 5′-ggtaccCCATATCACATAATTTAAA GCC-3′ (lower case shows restriction site for *Kpn*I) and cloned in the sense orientation into *Xho*I and *Kpn*I sites of pKannibal resulting in pKannibal/A plasmid. Subsequently the 3′ UTR was amplified with primers 5′-tctagaGGATGATGATGGTTAAGA-3′ (lower case shows restriction site for *Xho*I) and 5′-aagcttCCATATCACATAATTTAAAGCC-3′ (lower case shows restriction site for *Hind*III) and then cloned in the antisense orientation in *Hind*III and *Xba*I sites of pKannibal/A giving pKannibal/A-B. The napin promoter (Josefsson et al. 1987) was ligated into pCR2.1 as an *Xho*I–*Sac*I fragment. Then the *Xho*I–*Xba*I cassette carrying intron-interrupted inverted repeats of the *FAD2* 3′-UTR was excised from pKANNIBAL/A-B and subsequently cloned

into the respective sites of pCR2.1 vector (Invitrogen) behind the napin promoter. The resulting plasmid was named XC. A NOS terminator (Bevan 1983) amplified by PCR with primers 5′-tctagaGATCGTTC AAACATTTGGCAA-3′ (lower case shows restriction site for *Xba*I) and 5′-ggtcgacCGATCTAGTAA CATAGATGAC-3′ (lower case shows restriction site for *Sal*I) and subsequently as *Xba*I–*Sal*I fragment, was ligated with the *Xba*I–*Sac*I fragment from the XC plasmid into the respective sites of pRD400 (CLONTECH). The resulting plasmid was named XD (Fig. 2).

Isolation of the *Crambe abyssinica FAE* gene was performed as described previously (Mietkiewska et al. 2007). A *C. abyssinica* ORF was amplified by PCR with the primers: 5′-cccgggATGACGTTCCAT TAACGTAAAG-3′ (lower case restriction site for *Sma*I) and 5′-ggatccTTAGGACCGACCGTTTTGG-3′ (lower case restriction site for *Bam*HI). The napin promoter was amplified with primers 5′-gaattcAA GCTTTCTTCATCGGTG-3′ (lower case restriction site for *Eco*RI) and 5′-cccgggGTCCGTGTATGTTT TTAATC-3′ (lower case restriction site for *Sma*I). The NOS terminator was generated by PCR with the primers 5′-ggatccGATCGTTCAAACATTTGGCA A-3′ (lower case restriction site for *Bam*HI) and 5′-ga gctcCGATCTAGTAACATAGATGAC-3′ (lower case restriction site for *Sac*I). The napin promoter as an *Eco*RI–*Sma*I fragment, the *C. abyssinica FAE* as an *Sma*I–*Bam*HI fragment and the Nos terminator as a *Bam*HI–*Sac*I fragment were ligated into the *Eco*RI–*Sac*I sites of pSK$^+$, resulting in plasmid ZB. Subsequently the *Eco*RI–*Sac*I cassette was excited from the ZB plasmid and cloned into the respective sites of the XD plasmid resulting in plasmid XS (Fig. 2).

The final binary vectors XD and XS were electroporated into *Agrobacterium tumefaciens* cells strain GV3101 containing helper plasmid pMP90 (Koncz and Schell 1986). Plasmid integrity was verified by DNA sequencing following its re-isolation from *A. tumefaciens* and transformation into *E. coli*.

Plant transformation

Brassica carinata plants were transformed by the method of Babic et al. (1998). Transgenic plants were selected and analyzed as described by Mietkiewska et al. (2007).

Northern and southern analysis

Total RNA from *B. carinata* plant material was isolated as described by Lindstrom and Vodkin (1991). Twenty micrograms of RNA was fractionated on a 1.4% (w/v) formaldehyde-agarose gel and the gels were then stained with ethidium bromide to ensure that all lanes had been loaded equally (Sambrook et al. 1989). The RNA was subsequently transferred to Hybond N$^+$ membrane (Amersham Biosciences, Baie d'Urfe, Canada). A 0.5-kb probe containing the 3′ part of *FAD2* gene was generated by

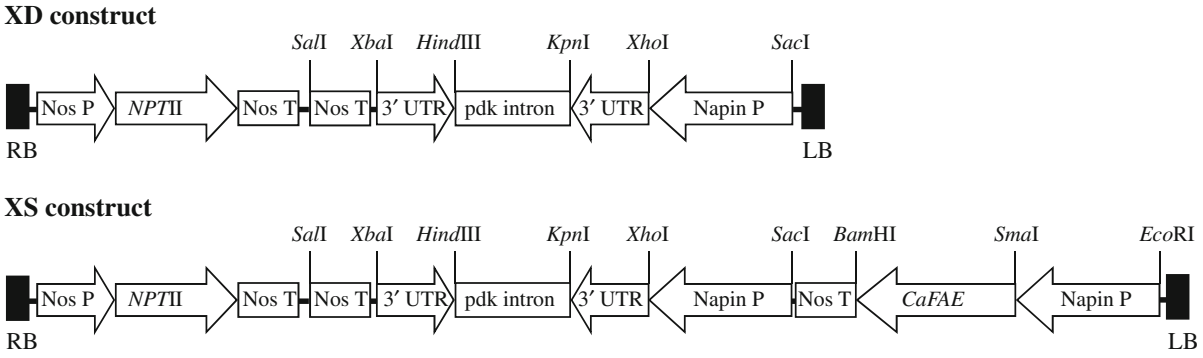

Fig. 2 Schematic diagram (not to scale) of the XD and XS constructs used to transform *Brassica carinata* plants. Both constructs, driven by napin promoter (Napin P), consist of an inverted repeat of a 142 bp fragment (3′-UTR) corresponding to the 3′-UTR of the *B. carinata FAD2* gene (GenBank accession no: DQ250814) separated by the intron of pdk (Wesley et al. 2001). The XS construct also contains the coding region of the *C. abyssinica FAE* gene (*CaFAE*). The neomycin phosphotransferase gene (*NPT*II) is driven by the NOS promoter (NosP). The T-DNA left border (LB) and right border (RB) are shown. The positions of the restriction enzyme sites used for the cloning are as indicated

PCR using primers 5′-GTCTGCTACGGTCTCT ACCG-3′ and 5′-TCATAACTTATTGTTGTACC AG-3′ and subsequently radioactively labeled with ^{32}P using a Random Primers Labeling kit (Invitrogen). Membranes were hybridized at 60°C overnight. The filters were washed once in 1× SSPE, 0.1% SDS for 15 min and in 0.1× SSPE, 0.1% SDS for 5–10 min at the temperature of hybridization. The blots were exposed to X-OMAT-AR film (Kodak, Rochester, NY, USA).

Twenty micrograms of *B. carinata* genomic DNA was digested with the restriction enzyme *Eco*RI, and the resulting fragments were separated on a 0.9% (w/v) agarose gel and transferred to Hybond N^+ nylon membrane via an alkali blotting protocol. For plants transformed with the XD construct, a 1.1-kb DNA fragment containing the napin promoter amplified by PCR using primers 5′-AAGCTTTCTTCATCGGTG-3′ and 5′-TCCGTGTATGTTTTTAATC-3′ was used as the probe. A 1.5-kb probe containing the coding sequence of *C. abyssinica FAE* was generated by PCR using primers 5′-ATGACGTCCATTAACGTA AAG-3′ and 5′-GGACCGACCGTTTTGGGC-3′ and was used for the analysis of plants transformed with the XS construct. The labeling and hydridization were as described above.

Lipid analyses

The total fatty acid content and acyl composition of *B. carinata* seed oils were determined by gas chromatography of the fatty acid methyl esters (FAMEs) with 17:0 FAME as an internal standard as described (Katavic et al. 2001; Taylor et al. 2002; Mietkiewska et al. 2007). The lipid class separation was carried out according to the method of Christie (1982). Polar and neutral lipids species were separated by TLC on Silica Gel 60 H plates developed 4 cm in diethyl ether, air dried and then developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v). Subsequently polar lipids were further developed in chloroform:methanol:acetic acid:water (25:10:3:1, v/v/v). TLC regions containing lipid species were scraped and samples saponified with 2 ml of 10% KOH in methanol at 80°C for 2 h. Following isolation of the free fatty acids, FAMEs were produced using 3 N methanolic HCl and extracted and analyzed by GC as described earlier (Taylor et al. 2002). Relative fatty acid compositions were calculated as the percentage that each fatty acid represented of the total fatty acids. An additional indirect method of assessing the cumulative effects of FAD2 activity during seed fatty acid synthesis through an oleic desaturation proportion (ODP) parameter was calculated as described by Stoutjesdijk et al. (2002).

Results and discussion

Fatty acid composition of preliminary transformants

Brassica carinata plants were transformed with two constructs: XD, targeted at the endogenous *FAD2* gene utilizing intron-spliced hpRNA-mediated gene silencing, and XS targeted at silencing the *FAD2* gene along with heterologous expression of the *Crambe abyssinica FAE* gene (Fig. 2). Wild type *B. carinata* contains two copies of both the *FAD2* and *FAE* genes. Eleven T_0 plants carrying the XD construct arising from seven independent transgenic lines, were identified that were both resistant to kanamycin and PCR-positive for the presence of the transgene. Thirty-six plants were transformed with the XS construct representing 20 independent lines (data not shown).

The fatty acid composition of T_1 seeds from individual plants was determined for all transformants. The best independent transgenic lines are shown in Fig. 3a. Seed specific silencing of the *FAD2* gene resulted in a significant reduction of the level of 18:2 from 19.0% in the wild type background to as low as 4.5% in line XD-4D. The strong reduction in the level of 18:2 was directly correlated with a significant increase in the proportion of oleic acid, up to 21.2% in the best transgenic line carrying the XD construct, compared to 5.7% in the wild type background. The increased pool of 18:1 was utilized by the endogenous microsomal elongation complex (FAE) and this led to the increase in the proportion of erucic acid to as high as 50.6% in line XD-5A, an 11% net increase, and a 26.5% proportional increase over wild-type levels of 40.0% 22:1. The high increase in erucic acid achieved in the current study through ihpRNA-mediated silencing of the *FAD2* gene is considerably greater than that reported by Jadhav et al. (2005), where transformation with the

FAD2 sequence in an antisense orientation resulted in a net increase in erucic acid of 6% in the best line, compared to the WT *B. carinata* seeds.

In order to study whether the increased pool of oleic acid can contribute to the higher accumulation of erucic acid obtained by heterologous expression of *C. abyssinica FAE*, we also designed the XS construct (Fig. 2). As expected, the pronounced silencing of the oleoyl desaturase resulted in large reduction in linoleic acid and concomitant increases in the proportions of oleic acid (Fig. 3b). The level of 18:2 in transgenic lines carrying the XS transgene was as low as 4.0% in XS-18A compared with 16.0% in WT. The increased pool of oleic acid in seeds of the XS transgenic plants was subsequently elongated with assistance from the heterologously-expressed *C. abyssinica* FAE which resulted in a net increase of the proportion of erucic acid in T_1 segregating seeds of up to 16%, a 42% relative increase compared to the wild type. Our current approach resulted in a higher accumulation of erucic acid than that formerly reported utilizing *C. abyssinica FAE* alone, which resulted in a net increase of 10% in the proportion of erucic acid in T_1 seed oils (Mietkiewska et al. 2007).

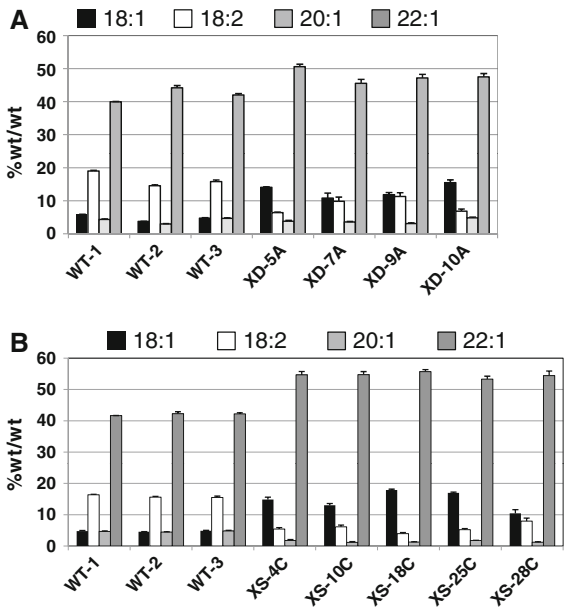

Fig. 3 Proportion of unsaturated fatty acids in seed oils from nontransformed *B. carinata* (WT-3 lines) and *B. carinata* T_1 independent lines transformed with (**a**) XD and (**b**) XS constructs. The values are the average ± SD of three determinations

The effect of gene silencing on oleate desaturation levels in seed from preliminary transformants

Oleate desaturase is highly active in developing seeds of wild type *B. carinata*, with 85% of 18:1 being converted to 18:2 and 18:3, for an oleic acid desaturation proportion (ODP) value of 0.85 (Table 1). Many transgenic T_1 plants carrying XD and XS constructs showed a considerable reduction in the ODP value, to as low as 0.39, indicating a profound (55%) down-regulation of oleate desaturation. Most transgenic plants have ODP values from 0.4 to 0.7 meaning that only 40–70% of oleic acid produced in developing seeds carrying the silencing constructs was converted to polyunsaturated 18-carbon fatty acids compared with 85% in non-transformed *B. carinata* seeds. As shown in Fig. 4 a positive correlation between the degree of *FAD2* gene silencing (expressed as ODP value) and the content of erucic acid was observed. In our study, as in others (Lu et al. 2002; Stoutjesdijk et al. 2002), all transgenic plants (100%) analyzed showed *some* degree of *FAD2* gene silencing (Table 1), a major advantage for this technique over other silencing methodologies wherein only some transformants display the expected phenotypic change. While the intron-spliced hpRNA-mediated (iHP) gene silencing technique used in the present study has not been a means to achieve a complete knockout of the seed oleate desaturase (see http://www.pi.csiro.au/RNAi/; Stoutjesdijk et al. 2002; P Waterhouse, personal communication), iHP did modify the expression of the *B. carinata FAD2*, with a much higher efficacy of gene silencing than we reported earlier using antisense or cosuppression approaches (Jadhav et al. 2005). In *A. thaliana* the highest efficiency of *FAD2* gene silencing was achieved using an iHP construct utilizing a 120-bp fragment of *FAD2* 3′-UTR (Stoutjesdijk et al. 2002). The presence of the intron in an iHP construct may result in increased or more stable transcript levels than in the nonintron-containing hpRNA constructs (Tanaka et al. 1990; Stoutjesdijk et al. 2002).

The stability and inheritance of ihpRNA-mediated gene silencing for lipid metabolism related genes has been shown (Stoutjesdijk et al. 2002; Liu et al. 2002);

Table 1 Frequency distribution of ODP value in seed of *Brassica carinata* (WT) and T_1 seed of *B. carinata* transformed with XD and XS construct

Construct	No. of lines	No. of lines with ODP value in the class					
		0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9
WT	3	0	0	0	0	0	3
XD	11	2	1	3	4	1	0
XS	36	0	9	11	15	1	0

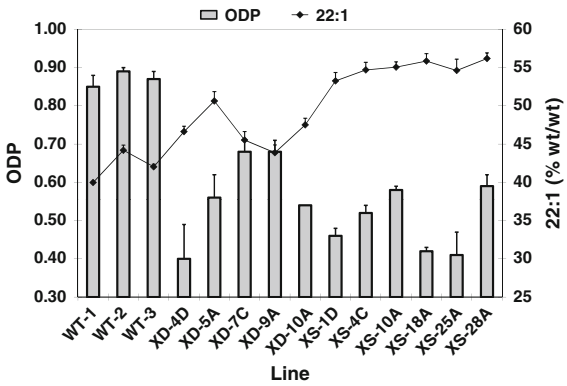

Fig. 4 Correlation of *FAD2* gene silencing activity expressed as an ODP value (gray bars) with erucic acid content (◆) in seed oils from nontransformed *B. carinata* (WT-3 lines) and selected *B. carinata* T_1 independent lines transformed with the XD and the XS constructs

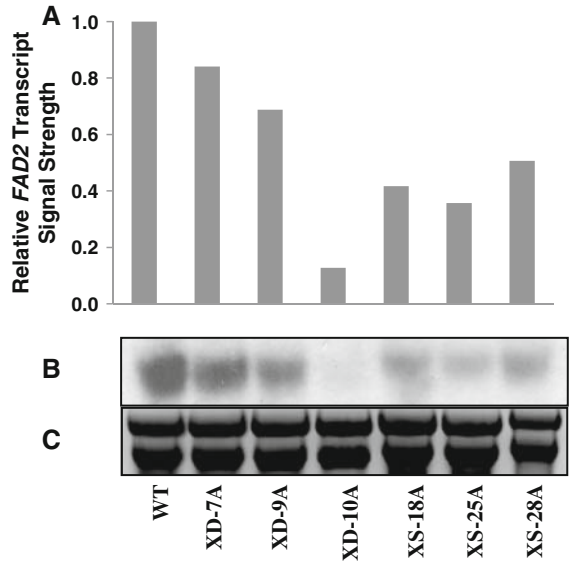

Fig. 5 Northern blot analysis of *FAD2* gene expression in nontransformed *B. carinata* (WT) and in T_1 transgenic seeds carrying the XD and XS constructs. Total RNA was isolated from mid developing seeds, blotted and probed with a ^{32}P-labeled 0.5-kb fragment of the *FAD2* gene. In the top panel (**a**), the bars indicate the relative hybridization signal strength (transcript intensity) with the labeled *FAD2* probe; signal intensities are integrated and background subtracted with Image J Image Processing and Analysis Software (http://rsb.info.nih.gov/ij/index.html). Signal strengths are reported relative to that in WT which is set at 1.0. The middle panel (**b**) is the actual northern gel hybridization result. The lower panel (**c**) indicates ethidium bromide staining of the ribosomal RNA bands, used to estimate equal loading of the lanes on each gel

specifically, two published papers on the application of an ihpRNA approach for silencing FAD2 (Yang et al. 2006) and DGAT (Zhang et al. 2005) were based on the results from T_0 plants. The high efficiency of gene silencing obtained through the use of ihpRNA-mediated PTGS makes this technique a very valuable contribution to practical trait modification in agricultural plants. This is particularly important for plants with low efficiency of transformation (Liu et al. 2002).

Effect of silencing construct on the *FAD2* mRNA level

Based on high oleic acid levels, the six best lines were selected to examine the *FAD2* mRNA level in transgenic seeds by northern analysis (Fig. 5). In all transgenic lines, the *FAD2* mRNA level decreased compared to that of the wild type (WT). The respective *FAD2* 3′UTR transgene copy numbers were: XD-7A (1), XD-9A (1), XD-10A (2); XS-18A (1), XS-25A (multiple), XS-28A (1). In the case of XS samples, since the *FAD2* 3′UTR and *Crambe FAE* are expressed in tandem between the left and right borders, the copy number refers to both the *FAD2* and *CrFAE* transgene copy numbers. The probe used for the XS Southerns was a *Crambe FAE.*

While there was not an absolute correlation between transgene gene copy number and *FAD2*

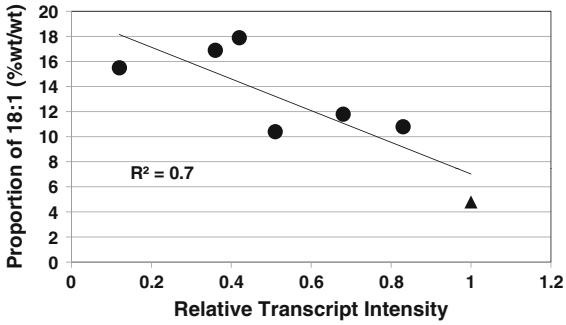

Fig. 6 Inverse relationship between relative *FAD2* transcript intensity and the proportion of 18:1 in total seed lipids in the XD and XS transgenics (●) and the non-transformed control (▲) *B. carinata* lines

transcript intensity, the two-copy XD-10A and the multiple copy XS-25A, did exhibit the stronger transcript suppression within their respective datasets. The results demonstrated a good correlation between the degree of transcript reduction induced by PTG silencing, and the increased proportions of the 18:1 in total seed lipids (Fig. 6). Similar trends have been reported from silencing *FAD2* gene in tobacco and *FAD2-1* in cotton (Yang et al. 2006; Liu et al. 2002).

Effect of silencing constructs on TAG composition in WT vs. XS-18A transgenic *B. carinata* seeds

Individual lipids extracted from seed tissue were analyzed for fatty acid composition in seeds of WT and of the best performing XS line 18A. In the seeds of XS-18A, both polar and neutral lipids showed an increase in the level of 18:1 compared with WT seeds. Among polar lipids (data not shown), the most pronounced increase of 18:1 was found in phosphatidylethanolamine (PE) and phosphatidylcholine (PC), as high as 6-fold and 3-fold, respectively. In PC from *FAD2*-silenced seeds, the increase in the level of 18:1 was accompanied by strong reduction in the level of palmitic acid (16:0). Similar changes in polar lipid class composition were reported for leaves and seeds of *FAD2* silenced tobacco plants (Yang et al. 2006). These phenomena were also found in an *Arabidopsis thaliana FAD2* mutant and in *FAD2*-silenced *Gossypium hirsutum* plants (Miquel and Browse 1992; Liu et al. 2002). As proposed by Yang et al. (2006) *FAD2*-silencing may have effect on de novo 16:0 synthesis. The profound increase of 18:1 with the concomitant decrease of 18:2 may affect the fluidity of membranes, which could result in down-regulation of 16:0 content by a mechanism which is not yet understood.

More importantly, as shown in Table 2, the 18:1 proportion in seed triacylglycerols (TAGs) increased by 3.5-fold compared to the WT seeds, at the expense of 18-carbon polyunsaturated fatty acids. The erucic acid proportion rose from 40% in WT, to 56% in the RNAi *FAD2* + *Crambe FAE* transgenic line XS-18A. This is the best result observed in transgenic manipulations of *B carinata* to enhance erucic acid content of its seed oil. Such prototypic results make *B. carinata* an increasingly-attractive alternative to HEA *B. napus* cultivars as an industrial oil crop.

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Table 2 Fatty acid composition of triacylglycerols from mature seeds of wild type *B. carinata* (WT) and XS-18A transgenic line

	Fatty acid composition (% w/w)								
	16:0	18:0	**18:1**	**18:2**	18:3	20:0	20:1	22:0	**22:1**
WT	3.5 ± 0.1	0.9 ± 0.1	**4.7 ± 0.1**	**15.8 ± 1.1**	16.3 ± 0.6	0.7 ± 0.1	4.7 ± 0.1	1.1 ± 0.1	**40.0 ± 1.1**
XS-18A	2.2 ± 0.3	0.6 ± 0.1	**17.6 ± 0.3**	**4.0 ± 0.1**	8.0 ± 0.2	0.5 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	**55.9 ± 0.7**

The values are the average ± SD of three determinations

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