

Characteristics of High α -Linolenic Acid Accumulation in Seed Oils

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Received: 6 March 2008 / Accepted: 3 June 2008 / Published online: 3 July 2008
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Abstract Modern diets are often deficient in ω -3 fatty acids and additional dietary sources of ω -3 fatty acids are useful. In order to investigate the molecular basis of the high accumulation of the ω -3 fatty acid, α -linolenic acid (18:3), in three different plants, flax (*Linum usitatissimum*), *Dracocephalum moldavica*, and *Perilla frutescens* ω -3 desaturase activity, transcript levels, and 18:3 in-vivo synthesis were examined. The 18:3 content was found to be higher at the later developmental stage of *D. moldavica* (68%) compared with *P. frutescens* (59%) and flax (45%) cotyledons. The 18:3 and 18:2 contents in both PC and TAG were determined during various stages of seed development for all three plants in addition to soybean (*Glycine max*). Northern blot analysis data of three different stages of *D. moldavica*, flax, and *P. frutescens* compared with moderately low 18:3 producers, soybean (*Glycine max*), and *Arabidopsis thaliana* and *Brassica napus*, (8–10% 18:3) at a stage of zygotic embryo development of high triglyceride synthesis showed that ω -3 desaturase mRNA levels were higher in all three high 18:3 producers, flax, *D. moldavica* and *P. frutescens*. This indicates that the high level of α -linolenic acid in TAG may be largely controlled by the level of ω -3 desaturase gene expression. However, the PC versus TAG fatty acid composition data suggested that along with ω -3 desaturase

other enzymes also play a role in 18:3 accumulation in TAG, and the high accumulators have a selective transfer of α -linolenic acid into TAG.

Keywords Fatty acid · Lipids · Omega-3 · Flax · Perilla

Abbreviations

ACP	acyl carrier protein
ALA	α -linolenic acid
DAG	diacylglycerol
DGAT	diacylglycerol acyl transferase
DHA	docosohexaenoic acid
EFA, EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FFAP, PC	phosphatidylcholine
PUFA	polyunsaturated fatty acid
TAG	triacylglycerol

Introduction

The diets of people in some countries come close to the apparent ω -3/ ω -6 fatty acid ratio of diet during human evolution: such as the traditional diets of Greece and Japan. In both countries the rate of death due to cardiovascular disease is among the lowest. It is now recognized that there is a need to return ω -3 fatty acids into the food supply, both for normal growth and development and for the prevention and management of chronic diseases [1]. Human beings evolved on a diet in which the ratio of omega-6/omega-3 EFA was about 1, whereas in the western diets the ratio is 15/1 to 16.7/1. Increased use of certain plant oils and modern day agriculture has led to a decrease in omega-3

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fatty acids and increases in omega-6 fatty acids [2–4]. Linoleic acid and α -linolenic acid are not interconvertible in animals and compete for the rate limiting delta-6 desaturase in the synthesis of long chain PUFA. Among the cardiovascular benefits of increased omega-3 fatty acids in the diet is the prevention of cardiac arrhythmias [4]. Already eggs high in omega-3 fatty acids are available on the market [5]. All these factors increase proportionally with an increase in the omega-3 fatty acid intake, including either ALA or EPA, but especially DHA.

Seed oils are composed primarily of triacylglycerols (TAGs), which are glycerol esters of fatty acids. The primary fatty acids in the TAGs of oilseed crops are 16–18 carbons in length and contain 0–3 double bonds. Palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) predominate. In this study we used soybean (*G. max*) as a moderate linolenic acid producer and three different high linolenic acid producers: flax (*Linum usitatissimum*) which has a high content of linolenic acid in the triacylglycerol (TAG), normally more than 45% of the total fatty acid content [6–8], *Perilla frutescens* oil contains 50–64% of 18:3 [9–11], and *Dracocephalum moldavica* contains 59–68% 18:3 [9, 12, 13].

Flax (*L. usitatissimum*) oil content ranges from 40 to 52% and 40 to 64% of this oil is linolenic acid depending on the flax variety [6–8]. *D. moldavica* contains 25–30% oil and 59–68% of it is 18:3 [9, 12, 13]. The oil content of *P. frutescens* is 35–45%, and contains 50–64% of 18:3 [9–11]. It is known that ω -3 fatty acids are synthesized from ω -6 fatty acid precursor membrane lipids in plastids and the ER by ω -3 desaturases [14].

The objective of this study was to investigate the biochemical basis of α -linolenic acid accumulation in developing seeds of flax (*L. usitatissimum*), *D. moldavica* and *P. frutescens* as high linolenate accumulators compared to the moderate accumulators soybeans and canola.

Materials and Methods

Plant Materials

Soybean (*G. max*) cv. 'Jack', *Brassica napus* cv. 'Westar', flax (*L. usitatissimum*) cv. 'NorLin', *P. frutescens* and *D. moldavica* plants were grown in the greenhouse at the University of Kentucky, Lexington. Dicot embryogenesis is divided into five general stages: globular, heart, cotyledon, maturation, and dormancy. By the end of the cotyledon stage and during maturation primary storage products including lipids accumulate in preparation for seed germination later [15]. In this study we focused on cotyledon to maturation stages and classified them to six different stages based on seed size, color, and weight. Seed

coats were carefully removed; embryo length and fresh weight were measured. Samples were frozen in liquid N₂, lyophilized and the dry weight was measured.

Fatty Acid and Lipid Analysis

The overall fatty acid composition of seed tissues of different cotyledon developmental stages of the investigated plants were analyzed by modifications of the procedure of [16]. About 1–10 mg lyophilized seed samples with added heptadecanoic acid (17:0) were placed into 2 mL of 2% (v/v) H₂SO₄ in methanol. The samples were ground finely, and then heated at 80 °C until the volume was reduced to approximately 0.5 mL (2 h). One and one-half milliliters of hexane containing 0.001% butylated hydroxytoluene (BHT) were added and the mixture was vortexed vigorously. The fatty acid methyl esters in the hexane layer were separated by gas chromatography on a Hewlett-Packard 0.25 mm I.D. × 0.33 mm × 10 m FFAP column and quantified using a flame ionization detector. A Hewlett-Packard 5890A gas chromatograph was programmed for an initial temperature of 140 °C for 1 min followed by increases of 12 °C/min to 210 °C and to 235 °C. The final temperature was maintained for 8 min. The injector and detector temperatures were 220 and 250 °C, respectively. Helium was used as the carrier gas with a flow rate of 10 mL/min. Total lipids were extracted from seed tissue as described by [17]. Individual lipids were separated by one-dimensional thin layer chromatography 20 × 20 cm TLC plate (LK60 silica gel 60 A°, Whatman) by the method of [18]. TLC plates were developed in two solvent systems. Phospholipids and glycolipids were separated in chloroform:methanol:water (65:25:4, v/v). Neutral lipids were separated in hexane:diethyl ether:acetic acid (100:100:2, v/v) [17, 19]. Lipids were located by spraying the plates with a solution of 0.005% primulin in 80% acetone, followed by visualization under UV light. In order to determine the fatty acid composition of individual lipids, ten µg of 19:0 was added to the silica gel of each lipid band before scraping, transferred to a tube containing 2 mL of 2% (v/v) H₂SO₄ in methanol, and fatty acid methyl esters were prepared and analyzed as described above. Radiolabeled 18:2 CoA was synthesized using ¹⁴C linoleic acid and co-enzyme A by the method described by [20]. The labeled ¹⁴C 18:2-CoA was fed to the embryos on a µL (0.0028 µCi/µL) per gram fresh weight basis and incubated for 90 min in light as described by [8]. After incubation the embryos were washed to remove unabsorbed radioactivity and crushed in chloroform/methanol (2:1) to extract lipids. Reverse phase chromatography was used to separate the 18:2 from 18:3. The free fatty acids were methylated by diazomethane and the esterified fatty acids by sodium methoxide. The samples along with the standards were

loaded on to a KV18 silica gel plate and ran in a solvent system consisting of acetonitrile/acetic acid/water (70:10:10). The image was developed in a phosphorimager. The standards were identified and thus used to identify the location of the radioactivity, by placing the plate into an iodine chamber.

RNA Isolation

RNA was isolated using the Trizol Reagent (Life Technologies, GibcoBrl). 50–100 mg seed tissues of different cotyledon developmental stages of tested plants were frozen in liquid N₂, homogenized in 1 mL Trizol reagent, transferred into microcentrifuge tubes and incubated for 5 min at room temperature. 0.2 mL chloroform per 1 mL of Trizol was added; tubes were vigorously shaken for 15 s and incubated at room temperature for 3 min. Samples were centrifuged at 12,000×g for 15 min at 2–8 °C. The upper aqueous phase containing RNA was collected in fresh tubes; RNA was precipitated with 0.5 mL of isopropyl alcohol and samples incubated at room temperature for 10 min. Samples were centrifuged at 12,000×g for 10 min, and the RNA pellet was washed once with 1 mL of 75% ethanol. Samples were vortexed and centrifuged at 7,500×g for 5 min. The RNA pellet was air-dried, then dissolved in RNase-free water and incubated for 10 min at 60 °C. The concentration of RNA was estimated by reading the absorbance at 260 nm. The 260/280 ratio of the RNA was 1.9–2.0. The quality of RNA was checked using 1.2% agarose gels. Samples were stored at –80 °C until used.

Northern Blotting

RNA samples (30 µg) were prepared by adding 2.0 µL of 5× formaldehyde gel-running buffer, 3.5 µL 12.3 M formaldehyde, 10 µL formamide, and incubated 15 min at 65 °C. The samples were chilled in ice for 3 min. Samples were immediately loaded on a pre-run denaturing 1% agarose-formaldehyde gel, the electrophoresis was carried out in the presence of 1× formaldehyde gel-running buffer. The gel was washed with 0.05 N NaOH for 20 min, rinsed with RNase-free water, followed by 10× SSC for 45 min. Blotting and transfer of RNA onto the 6× SSC pre-soaked nylon membrane was carried out according to the standard procedure [21]. RNA was fixed to the Zeta-probe nylon membrane by UV cross-linking (UV stratalinker 1800, Stratagene). The *B. napus* ω-3 desaturase probe was labeled with [α -³²P] dCTP using the random priming method. Pre-hybridization and hybridization were carried out at 42 °C following the instructions for using the Zeta-Probe Membrane (Bio-Rad) as outlined by the manufacturer. After hybridization a PhosphorImager system

(Molecular Dynamis, 445 SI) was used to visualize the bands obtained. The radioactive probes were stripped by washing the blots three times with 0.1% SDS in 2× SSC at 95 °C, 10 min each.

Results

Fatty Acid Composition in Different Oil Seeds of Moderate and High Linolenate Accumulators During Maturation

The percentage of total fatty acids in flax seeds at different cotyledon developmental stages were analyzed (Fig. 1). Linolenic acid (18:3) accumulated during seed

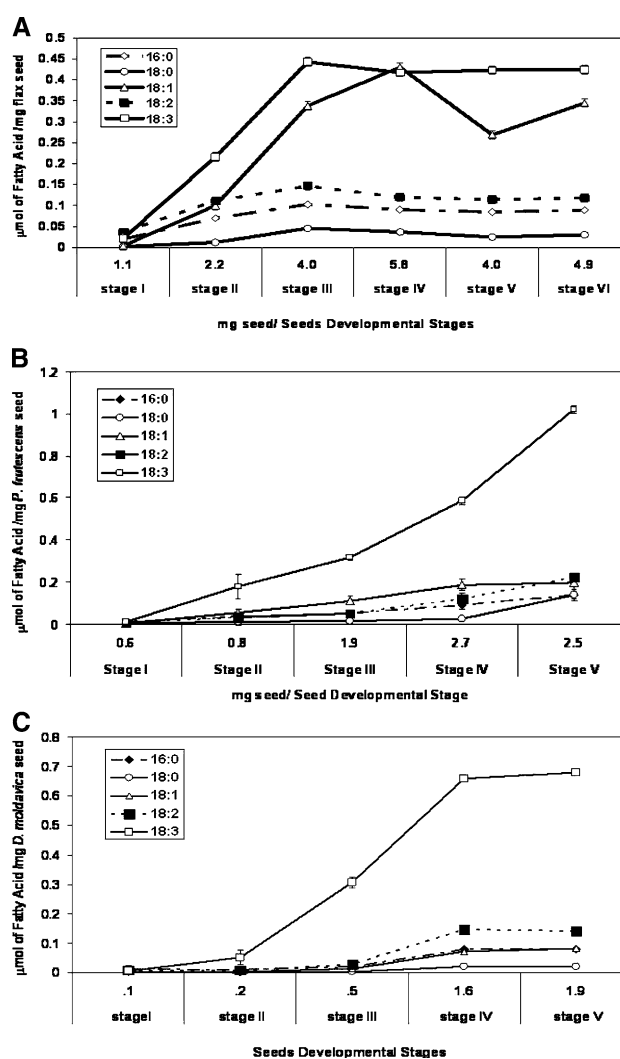


Fig. 1 Accumulation of fatty acids in developing flax seeds (a), *P. frutescens* seeds (b), *D. moldavica* seeds (c). µmol of fatty acid/mg seed dry weight were calculated during different stages of cotyledon development. Data points are the means of six replications ± standard errors

development and reached 46% by the end of maturation, oleic acid increased during maturation until stage IV at 40% and then become steady until the end of the maturation. 18:2 was found to be 45% at the first stage and dropped dramatically during the rest of the developmental stages to reach 10% by the end of the maturation process. Also 16:0 dropped from 25% at the beginning of the cotyledon maturation stage to 10% by the end of seed maturation.

The synthesis of 18:3 takes place rapidly at the beginning of cotyledon development in *P. frutescens*. The level of 18:2 declines with the increase in 18:3 while the 18:1 content slightly increased during seed maturation but its level remained relatively lower than the case of flax seed. This suggests that Δ -12 desaturase is more efficient in desaturating 18:1–18:2 in *P. frutescens* than in flax. *P. frutescens* seed has a high content of α -linolenate (57%) [10, 22, 23] and other fatty acid contents were also similar to reported values. The percentage ratio for both 18:2 and 18:3 in *P. frutescens* seed during maturation indicates that ω -3 desaturase is very active at the beginning of cotyledon development. However, the 18:3 content reached a steady state at the third stage and remained constant thereafter until the end of seed maturation (Fig. 1). The amount of 18:3 ($\mu\text{mol}/\text{mg}$ dry weight) increased during cotyledon developmental stages to reach 1.15 $\mu\text{mol}/\text{mg}$ dry weight at the maturation stage.

The accumulation of 18:3 in *D. moldavica* increased gradually to 68% by the end of seed maturation (Fig. 1). The 18:2 and 18:1 levels are lower at maturity. This suggests that both Δ 12 and Δ 15 (ω -3) desaturases are very active in desaturation of 18:1 and 18:2 and are largely responsible for the high level of 18:3 in *D. moldavica*. The accumulation of other FA including 18:1 is minimal as in *P. frutescens* unlike the accumulation of 18:1 in flax during seed maturation. In all high 18:3 accumulators, the linolenic acid content starts at a moderate level. The percentage of 18:3 is higher at a very early cotyledon developmental stage (first stage) of *P. frutescens* (35%) compared with flax (25%) and *D. moldavica* (15%). α -Linolenic acid gradually accumulated in developing cotyledons of all three plants. However, 18:3 content was found to be higher at the late mature developmental stage of *D. moldavica* (68%) compared with *P. frutescens* (59%) and flax (45%) cotyledons under our greenhouse growth conditions (Fig. 1).

The ratio of 18:2 and 18:3 in TAG in developing soybean seeds is the opposite to that in the three high 18:3 accumulators previously mentioned, which suggests that the level of desaturation of 18:2 into 18:3 decreases in soybean as the seeds mature (Fig. 2). The unsaturated fatty acid levels in PC remain almost constant throughout seed maturation. The increase in lipid content in soybean seed is

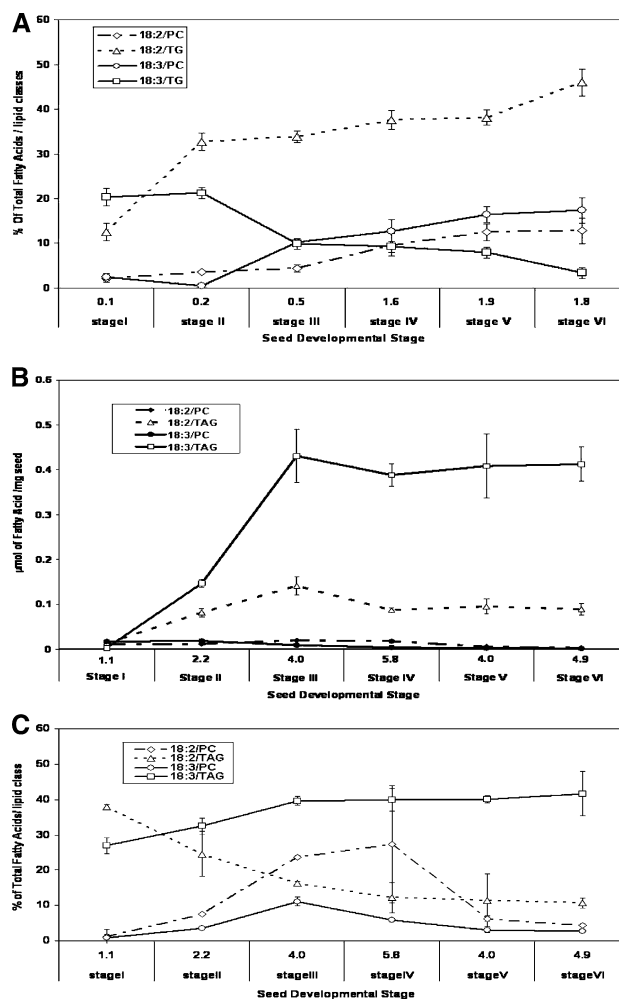


Fig. 2 Linoleic and linolenic acids in PC versus TAG in developing soybean seeds (a), Flax seeds (b), *P. frutescens* seeds (c)

accompanied by a shift in lipid composition from PC (membrane lipids) to TAG accumulation (storage lipids) during seed maturation [24, 25]. Figure 2 shows the amount of 18:3 in flax seed increases during cotyledon maturation stages in TAG in comparison to the 18:3 content in PC.

The level of 18:3 in *P. frutescens* TAG is much higher than in PC, also compared with flax (Fig. 2) the 18:2 levels in *P. frutescens* is lower in PC, which agree with the fact that 18:3 level in TAG of *P. frutescens* seed (Fig. 2) is much higher.

A slightly higher 18:3 amount was found in PC of flax seed in the first three stages of cotyledon development compared to the amount of 18:3 in PC of *P. frutescens* seeds (Fig. 2). However, the percentage of 18:3 in *P. frutescens* PC was slightly higher (6%) in the first two stages compared to the first two stages in flax PC (0.9–3%). Unlike TAG, the 18:3 content was low in PC throughout seed development in flax and *P. frutescens* (Fig. 2). An

increase in 18:3 content was observed in TAG of developing seeds in both flax and *P. frutescens*, however, flax seeds showed a gradual increase of 18:3 in TAG throughout all stages of development, while on the other hand a dramatic increase of 18:3 in TAG of *P. frutescens* seeds was observed during the later stages of development (from 0.15 $\mu\text{mol}/\text{mg}$ seed at the second stage up to 0.9 $\mu\text{mol}/\text{mg}$ seed at seed maturation). It was found that the second stage of cotyledon development is the optimum stage for monitoring ω -3 desaturase activity so this stage was used for northern blots.

The 18:3 levels are much higher in TAG compared to its level in PC in *D. moldavica* developing cotyledons while the level of 18:2 is lower in TAG but in PC the amount of 18:2 ($\mu\text{mol}/\text{mg}$ seed) is relatively higher during second to third stages of cotyledon development (data not shown). The 18:2 level in PC dropped by the end of maturation of the seed.

ω -3 Desaturase Activity

To investigate whether higher ω -3 desaturase activity is responsible for the higher synthesis of 18:3 fatty acid, which in turn is incorporated in to TAG in higher amounts, the ω -3 desaturase of flax and two moderate accumulators *Arabidopsis* and soybean were assayed. Feeding studies with ^{14}C labeled 18:2-CoA showed that developing embryos of flax have higher ω -3 desaturase activity compared to *Arabidopsis* and soybean (Fig. 3). Stage II flax seeds have much higher activity compared to the stage V flax seeds. During stage II 18:3 accumulation is linear and in stage V embryos 18:3 reaches a plateau (Fig. 1).

Microsomal ω -3 Desaturase Transcript Levels

The ω -3 desaturase transcripts levels in stage II flax embryos are much higher compared to soybean embryos of both stages and stage V flax embryos (Fig. 4). These results were also in line with 18:3 fatty acid accumulation in flax seeds (Fig. 1) and also indicated by the ω -3 desaturase

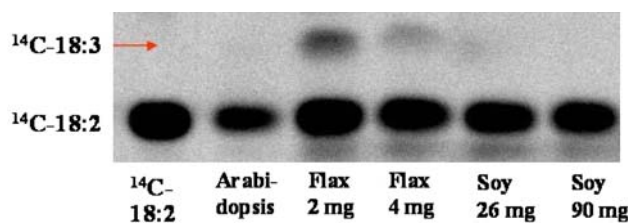


Fig. 3 ω -3 desaturase assays: ^{14}C -18:2 CoA was fed to developing embryos of high 18:3 accumulating flax and moderate 18:3 accumulators soybean and *Arabidopsis*. Lipids were extracted, methylated, and separated on a reverse phase C18 TLC plate and then read on a phosphorimager

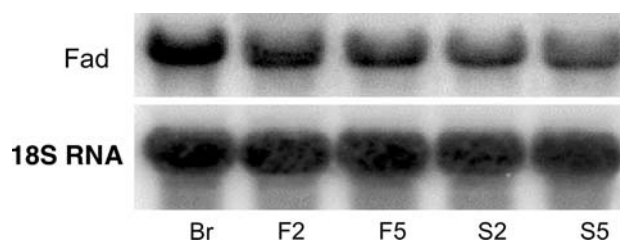


Fig. 4 Northern blot analyses of ω -3 desaturase expression in developing embryos of flax and soybean. RNA was extracted from: *B. napus* (Br), the second and fifth developmental stage of flax cotyledons (F1, F5) and the second and fifth developmental stage of soybean cotyledons (S2, S5). (Above) *B. napus* ω -3 desaturase (FAD3) cDNA was used as a probe to check the expression of FAD3 gene (Below) to confirm the amount of RNAs loaded were equal 18S ribosomal DNA was used as probe to measure 18S rRNA expression. Equal RNA loading was also confirmed by measuring RNA concentration spectroscopically at 260 nm

activity assay (Fig. 3). The activity assay results show that stage II flax embryos had much higher activity than both stages of soybean embryos and stage V flax. Northern blot analysis data also show developing embryos of *P. frutescens* have much higher ω -3 desaturase transcript levels than soybean and *Arabidopsis* (Fig. 5). When three different stages of *D. moldavica*, *P. frutescens* and flax, were compared with the moderately low 18:3 producers (8–10%), soybean and *Arabidopsis*, and *B. napus*, ω -3 desaturase mRNA levels were also higher in all three high 18:3 producers, flax, *D. moldavica* and *P. frutescens* compared with the moderately low 18:3 producers (figure not shown for *D. moldavica*). However among high 18:3 producer plants, the ω -3 desaturase transcript level was found to be much higher in *D. moldavica* than in *P. frutescens*, which in turn has a higher transcription level than flax (Fig. 5).

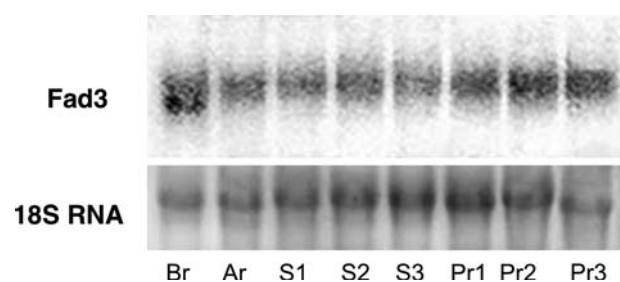


Fig. 5 Northern blot analyses of ω -3 desaturase gene expression in developing embryos of *B. napus*, *Arabidopsis*, soybean and *P. frutescens*. RNA was extracted from *B. napus* (Br), *Arabidopsis* seed (Ar), soybean seed cotyledon developmental stages 5–6 mm (S1), 7–9 mm (S2), 9–11 mm (S3), Pr1, Pr2, Pr3, are *P. frutescens* cotyledon at developmental stages (based on size, color, and seed weight) 1, 2 and 3?. (Above) RNAs were blotted on a nylon membrane and probed with *B. napus* ω -3 desaturase cDNA to check the ω -3 desaturase gene expression (Below) To confirm the amount of RNAs loaded were equal 18S ribosomal DNA was used as probe to measure 18S rRNA expression. Equal RNA loading was also confirmed by measuring RNA concentration spectroscopically at 260 nm

The lipid content and compositions of soybean seeds changes considerably as the embryo progress from the early stages of development to the maturation stage [26]. In soybean the level of 18:3 is higher at the beginning of the cotyledon maturation stage and begins to decline toward the late maturation stages while the 18:2 concentration increases. The opposite is found in all high 18:3 accumulators, with levels of 18:3 increasing during the maturation stages while the 18:2 levels decreased.

In flax (Fig. 3), ω -3 desaturase is highly active in converting 18:2-CoA to 18:3 compared to *Arabidopsis* and soybean.

Discussion

During the first three stages of cotyledon development, in all high 18:3 producers, linear increases in 18:3 levels were found. This suggests that these stages have the highest ω -3 desaturase activity. The 18:1 levels in both flax and *P. frutescens* (Fig. 1 and 2) suggests that Δ -12 desaturase is more active in desaturating 18:1–18:2 in *P. frutescens* than in flax. In *D. moldavica* 18:3 levels was very high compared with other fatty acids levels suggesting that lipid metabolism in this plant is optimally adjusted for 18:3 accumulation. The level of 18:3 in *P. frutescens* TAG is much higher than in PC, also compared with flax (Fig. 2) the 18:2 levels in *P. frutescens* is lower in PC, which is consistent with the high 18:3 level in TAG of *P. frutescens* seed (Fig. 2). This suggests that desaturation of 18:2–18:3 by ω -3 desaturase is more efficient in *P. frutescens* than in flax, which in turn is much higher than in soybean.

The changes in lipid concentration are accompanied by a shift in lipid composition from polar lipids to storage lipids [24]. In soybean the 18:2 levels in TG increase while 18:3 levels decrease. Acyltransferase enzymes catalyze the incorporation of fatty acids into the glycerol backbone to produce different glycerolipids classes including PC and TG [25, 27–29], so that from our data (e.g., Fig. 2) and the selective accumulation of 18:2 in TG of soybeans we can suggest that acyltransferases are selective with 18:2 over 18:3 in soybean seed. However, the absolute amount (μ mol/mg seed) of 18:3 in high producing plants suggests that the incorporation rate into TG is very high which in turn indicates that acyltransferases may selectively incorporate 18:3 into storage lipids.

Northern blot data shows that the ω -3 desaturase gene expression level is higher in high 18:3 producing plants compared to low producers. This indicates that the level of ω -3 desaturase gene expression may largely control the level of α -linolenic acid in TAG, although as mentioned above acyltransferases also appear to be involved. High accumulation of 18:3 correlates with ω -3 desaturase

mRNA levels which suggests transcriptional control of fatty acid desaturase genes [30, 31]. However, the PC versus TAG fatty acid composition data suggest that ω -3 desaturase is not the only factor in 18:3 accumulation in TAG, and the high accumulators appear to have a selective transfer of α -linolenic acid into TAG which in turn suggested that certain acyltransferases or transacylases may play an important role in 18:3 accumulation in triacylglycerols. Another possible factor is the speed of flux from phospholipid to TAG as this can compete with 18:2–18:3 desaturation. It was shown in flax cultivars AC Emerson and Vimy that diacylglycerol acyltransferase (DGAT) prefers 18:3-CoA as substrate [32]. The data from both lipid analysis and Northern blot highly suggest that the reduced linolenic acid content in soybean compared with high 18:3 accumulators is mainly due to the lower transcript level of ω -3 desaturase. Byrum et al. [33] found that the reduced 18:3 concentration in the soybean genotype A5 was at least partially the result of a microsomal ω -3 desaturase gene with a reduced activity. Collectively, these results indicate that one can obtain *B. napus*, soybeans or other oilseed crops that accumulate high ω -3 fatty acid levels simply by increasing expression of the ω -3 desaturase gene by use of a strong promoter or trans-activation. Acyltransferases with specificity for 18:3 are likely to be needed for very high ω -3 fatty acid accumulation, for example, >80%.

Acknowledgments This work was supported by a graduate fellowship from Ain Shams University in Cairo Egypt to Mohammed Abdel-Reheem, the United Soybean Board and the KY Agricultural Experiment Station.

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