Biochemistry of high stearic sunflower, a new source of healthy fats.

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Abstract

Fats based on stearic acid could be a healthy alternative to existing oils especially hydrogenated fractions oils or palm, but only a few non-tropical species produce oils with these characteristics. In this regard, newly developed high stearic oilseed crops could be a future source of healthy fats and hard stocks rich in stearic and oleic fatty acids. These oil crops have been obtained either by breeding and mutagenesis or by suppression of desaturases using RNA interference. The present review depicts the molecular and biochemical bases for the accumulation of stearic acid in sunflower. Moreover, aspects limiting the accumulation of stearate in the seeds of this species are reviewed. This included data obtained of the characterization of genes and enzymes related to fatty acid biosynthesis and triacylglycerol assembly. Future improvements and uses of these oils are also discussed.

Key words: Oil crops, Helianthus annuus (sunflower), High stearic acid mutants, Lipid metabolism, Regulation of stearate levels.
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1. Introduction

1.1 Generalities

Sunflower (Helianthus annuus L.) is a plant of the family of the Compositae (Asteraceae), belonging to the genus Helianthus. This plant is an annual crop, easily recognizable by having a large yellow inflorescence consisting in a circular head or capitulum containing small flowers give place to achenes which contain a kernel that is rich in oil. This plant was domesticated and cultivated by natives from Central and North America, being introduced into Europe by Spanish explorers in the sixteenth century. The cultivation of sunflower was extended all over the world during the nineteenth and twentieth centuries, being today the fourth largest vegetable oil product (after palm, soybean and rapeseed) and an important agricultural commodity.
Today the main centers of production of sunflower are the USA, Ukraine and Argentina.

Sunflower is cultivated in temperate climates (temperature range between 20 and 25 °C). This plant grows better in dry climates with high solar irradiation and deep soils in which it is able to develop its long root system. The seeds of sunflower are produced within an achene and consist of a shell composed mainly of lignin and cellulose material and the kernel, which accounts for 80% of the total weight of the seeds and is rich in oil (up to 55%). The final oil content of sunflower seeds is around 50% w/w [1]. This oil is rich in linoleic acid, which accounts from 48 to 74% of the total fatty acids of the oil. It contains low levels of saturated fatty acids, mainly palmitic and stearic acids and, unlike other seed oils such as soybean and rapeseed, negligible amounts of linolenic acid. The relative amount of linoleic compared to oleic acid is very variable and is related to temperature regulation of endogenous desaturases [2,3].

1.2 Sunflower biotechnology

As in the case of other oil crops, there has been increasing interest in manipulating the fatty acid composition of sunflower oil to produce oils with new properties and better performance for different applications. In this regard, sunflower has been affected by the biotechnology revolution that has taken place in the last 20 years, but in a way that is different to other species. Thus, oil crops like rapeseed or soybean have been subject to many modifications and improvements through the application of genetic engineering. These manipulations involved the transfer of genes from other species or modification of the expression levels of endogenous genes by means of genetic transformation [4]. This has produced a variety of new lines with improved agronomical traits like resistance to herbicides and different oil compositions, varying from highly saturated oils to oils enriched in n-3 very long chain fatty acids [5-8]. This revolution has taken place in a climate of some public controversy (especially in Europe) where there has been concern of the potential impact of genetically modified products (GMOs) on human health and natural ecosystems. Thus, in the labelling of food in Europe and in many places of Asia it is necessary to declare the presence of transgenic material in the corresponding formulations. In this regard, sunflower has
not yet arrived into the GMO era. The reason is that sunflower is recalcitrant to transformation by means of Agrobacterium infection and is a plant very difficult to regenerate from cell cultures. Few advances have been on transformation either made by floral dip or embryo infiltration, so there are no transgenic sunflower lines for commercial exploitation at the present time. However, these facts haven’t restricted the improvement of sunflower lines using other techniques. Thus, sunflower is a plant easy to mutagenize by both physical and chemical methods. So, excellent results have been reported by using ethylmethane sulphonate or sodium azide to produce mutagenized populations of sunflower seeds [9,10]. These chemical mutagens promote point mutations during DNA duplication. Furthermore, sunflower can be also be mutagenized by ionizing radiation (X- or gamma-rays), which are responsible for the breakdown of large fragments of DNA, which causes deletions and mutations during the process of DNA repair. The population of mutagenized seeds can be later screened by TILLING [12] or, in the case of fatty acid compositional mutants, they can be found by direct GC analysis using half seeds (a nondestructive screening method in which the distal part of the seed is submitted to analysis and the apical part is used for propagation). Moreover, breeding of sunflower is eased by the simple diploid genetics of this plant. Another positive aspect of sunflower breeding is the relatively high genetic variability and the existence of several wild species of the genus Helianthus that could be a source of traits of interest to be transferred to commercial sunflower hybrids [11,13]. The breeding of sunflower, combined with the techniques of mutagenesis, have produced lines of sunflower with higher oil content, increased resistant to pests, dryness or salinity, as well as of mutants with altered fatty acid composition in their seeds. These improve agricultural, nutritional and technological performance of sunflower (and its oil) while the crop is not a GMO and, therefore, should be acceptable to a sceptical public (see above).

1.3 Sunflower mutant lines

In the last two decades many new lines of sunflower with modified fatty acid composition have been produced. The most successful development has been the production of high or ultra-high oleic mutants [14-16], which produce oils with fatty acid compositions similar to olive oil (Table 1, the basis of the healthy Mediterranean
Furthermore, the high levels of oleic acid make this oil more stable than most seed oils used for frying and storage. So, the cultivation and consumption of high oleic sunflower oil increases yearly all over the world with high acceptation both to industry and consumers. However, more recent programs of sunflower improvement have produced a second generation of modified sunflower oils in which their fatty acid composition differed much more from that in regular sunflower (Table 1). These mutants display high levels of saturated fatty acids, displaying very different physical properties and having a high potential for industrial applications [17,18]. The compositions of some of them are depicted in Table 1, where typical compositions of high stearic and high palmitic sunflower mutants are shown. In a high linoleic background these oils can reach as much as 30% of either stearic or palmitic fatty acids which, importantly, increases the solid fat content of such oils in a range of temperatures from 5 to 20°C. In this regard, there is a deficiency of hard stock fats in the world because most currently-used vegetable oils are liquid. Solid fats are necessary for many industrial formulations including margarines, shortenings, fillings or confectionary [19]. These fats can be prepared from vegetable oils by hardening through hydrogenation. However, this process causes the production of unhealthy trans-fatty acids as a by-product [20]. The other alternative is based on the use of palm oil. Palm oil is rich in palmitate and has been extensively used for human nutrition. It is also a very productive crop [19,21]. However, the increased cultivation of palm trees in Indonesia is causing destruction of broad areas of tropical forest (accompanied by smoke pollution) giving ecological concerns to the production of these fats. In this regard, fats enriched in stearic acid, like high stearic sunflower, seem to be a promising alternative for a sustainable and healthy source of solid fats. However, the original high stearic sunflower lines also contain high levels of linoleic acid, which diminishes its stability and solid content at high temperatures. So, the high stearic phenotype was transferred to high oleic (HSHO) lines to obtain the high stearic-high oleic mutant, which is being commercialized under the brand Nutrisun oil ©. The high stearic-high oleic oils can be fractionated to produce fats and butters with high levels of solids and different melting profiles [22,23] that can be used in a broad variety of food formulations, including fillings, spreads, coatings and confectionary products [24]. Furthermore, these sunflower-derived fats, rich in stearic and oleic acids, may be
considered healthier than those oils that are being used now for the same purposes such as hydrogenated fats. The HSHO oil has been also demonstrated to be an alternative to palm oleins for industrial frying, with good stability and a high vitamin content. The use of sunflower for the production of hardstock fats for industry has the extra advantages of avoiding any use of GMO, local production in temperate countries and the use of a crop that is not demanding in terms of agronomy.

The production of lines of HSHO sunflower with higher contents of stearic acid and better agronomic yield is going to be important in the future to make this crop more competitive. In so doing, an extensive characterization of the pathway of synthesis of fatty acids and of triacylglycerols has been carried out in sunflower seeds in order to find the aspects limiting the accumulation of stearic acid in the oil of sunflower. Here we review recent advances in these aspects.

2. Intraplastidial lipid synthesis

Sunflower are white seeds, which means that they are not able to photosynthesise during their development, a process that would help to recycle the CO₂ produced by cell respiration and so augment the overall carbon efficiency of oil synthesis [25]. As a sink organ, sunflower seeds receive all the carbon, energy and reducing equivalents from photosynthates exported via the phloem. Carbohydrates imported into the seeds, in the form of sucrose are degraded by glycolysis. So, the intermediates from this pathway (phosphoenol pyruvate (PEP) or pyruvate) are imported into the plastids where they are metabolised to give acetyl-CoA, the main precursor for the synthesis of fatty acids [25-27]. Acetyl-CoA is converted to malonyl-CoA and then to malonyl-ACP which serves as the two-carbon donor for elongation cycles catalyzed by the fatty acid synthase (FAS) complex. These cycles involve consecutive reactions of condensation, reduction, dehydration and a second reduction and depend on the availability of reducing equivalents coming mainly from the pentose phosphate pathway. The final products of the FAS complex are palmitoyl-ACP and stearoyl-ACP, which are the precursors of most plant fatty acids [28-29]. Sunflower seeds like those of most plants produce oils rich in C18 fatty acids, which means that they possess high activity of
condensing enzyme II (β-ketoacyl-ACP synthase (KAS) II) responsible for the elongation of palmitoyl-ACP to stearoyl-ACP, which thus determines the final ratio of C18/C16 fatty acids. Stearoyl-ACP is also the substrate for the first desaturation reaction in fatty acid synthesis. This step is catalyzed by a plastidial stearoyl-ACP desaturase (SAD). The desaturase specifically introduces a double bond in the Δ9 position of the acyl moiety of saturated acyl-ACPs. It is a mixed function oxygenase that removes two hydrogen atoms from the acyl carbon chain and two from reduced ferredoxin that are finally transferred to a molecule of oxygen, yielding two water molecules [30].

2.1 Stearoyl-ACP desaturase and high stearic trait.

The primary factor responsible for the high stearic phenotype in sunflower is a decrease of SAD activity. Genetic studies indicated that the high stearic trait was caused by two mutations [31, 32]. One of them affected one of the SAD sunflower genes and was responsible for most of the increase in stearate content. The other mutation also enhanced the level of stearic acid but the gene affected has not been identified yet. The SAD enzymes are very important within plant lipid metabolism, since they broadly determine the degree of unsaturation of most fatty acids present in the plant glycerolipids. Thus, there was a direct relationship between the content of linoleic acid and the level of stearic acid in sunflower, suggesting that it was regulation of the SAD enzyme activity which caused the major changes in the fatty acid composition of the oil. Fig. 1 shows that the decrease of the linoleic acid content in different mutant lines of sunflower involved a concomitant decrease of the level of stearic acid. So, the lines with a high oleic background tended to display lower stearate contents than lines with higher contents of linoleic acid [33]. This result suggests that regulation of the SAD genes may depend on the level of unsaturation of the fatty acids that are being synthesized. So, the very low levels of linoleate found in the high oleic Pervenets mutation may induce changes in membrane properties that tended to increase the level of unsaturation by activating desaturases such as SAD enzymes. This mechanism of regulation of the desaturation level of oilseeds has not been investigated to date but is an interesting field for future research. Moreover, the
decrease of SAD activity in sunflower seeds also caused a decrease of the seed oil content, although it did not affect germination. This decrease in the oil content was first thought to be caused by restrictions in the metabolic flux at the level of SAD activity, but later results suggested that this was an effect caused by restrictions in the flux at several points in the TAG synthesis pathway.

2.2 Acyl-ACP thioesterases

The pathway of plastid-localised elongation and desaturation of acyl chains is terminated by hydrolysis of the acyl-ACP derivatives through the action of acyl-ACP thioesterase enzymes [28]. These enzymes release an ACP molecule that is recycled for fatty acid synthesis and an unesterified (free) fatty acid that is exported out of the plastid to be esterified to CoA on the envelope and so used in the synthesis of glycerolipids. Acyl-ACP thioesterases are plant-specific enzymes that can be classified within two big gene families named FatA and FatB [34]. A notable characteristic of acyl-ACP thioesterases is their substrate selectivity, which determines the acyl-moiety that is released from the intraplastidial pool and, therefore, has an important impact on the final composition of the oil. The FatA and FatB thioesterases display a high degree of divergence that is reflected in their different selectivity [35]. Thus, FatA thioesterases display very high selectivity towards oleoyl-ACP, indicating that it is responsible for much of the export of de novo synthesized oleate which is then used in the endoplasmic reticulum for glycerolipid synthesis. FatB, on the contrary, also has high activity towards saturated fatty acids, mainly palmitic acid, and is, therefore, responsible for a large proportion of the saturated fatty acid present in plant glycerolipids [36]. In sunflower only one copy coding for each of the FatA and FatB genes has been cloned and characterized [37]. It is very likely that these two were the only copies present in the plant, but this will have to be confirmed by sunflower genome sequencing. The FatA from sunflower (HaFatA) was phylogenetically very close to the gene in Carthamus tinctorius, whereas, the sunflower FatB (HaFatB) was far from these forms in the cluster shown in Fig. 2, being more similar to the gene found in Capsicum annuum. Both enzymes are chloroplast-targeted proteins and carry
transit peptides in their N-terminal domains. As with the other enzymes of these families HaFatA was a soluble protein, whereas HaFatB showed a membrane anchorage domain, suggesting a stromal localization for the former enzyme and a possible binding of the latter to plastid membranes.

The enzyme HaFatA was expressed in E. coli after removing the transit peptide and adding a HIS-tag to enable its purification by means of Ni-NTA technology. This protein was expressed at high levels in the soluble phase of the bacteria and, once purified, was kinetically characterized [37]. It displayed very high $V_{max}$ values for oleoyl-ACP like most FatAs described in plants, but much lower activity towards saturated derivatives (around 2% of the $V_{max}$ value found for oleoyl-ACP). Furthermore, the rate of degradation of palmitoleoyl-ACP was moderate yielding $V_{max}$ values around 20% of that for oleoyl-ACP. The $K_m$ values of this enzyme were similar for all substrates, and were in the low micromolar range. The catalytic efficiency of this enzyme was high when compared with those values showed by thioesterases characterized from other species like castor bean or Arabidopsis (Table 2) indicating that HaFatA is a highly active enzyme, able to support high levels of metabolic flux though the pathway of fatty acid synthesis.

The enzyme HaFatB has also been characterized. The purification of this protein from a prokaryotic host was more difficult because it accumulated in inclusion bodies. The removal of the membrane domain of the enzyme prevented the accumulation of the protein in the particulate phase but high levels of expression of the gene induced lethality in bacteria. So it was expressed at low temperature and IPTG concentrations for characterization. The enzyme displayed high selectivity towards palmitoyl-ACP, but with high activity also towards oleoyl-ACP and lower activity towards stearoyl-ACP. The relative contribution of both thioesterases to the total flux of fatty acids exported from the plastids in developing seeds could be inferred from the relative expression of these enzymes. Thus, HaFatA is expressed up to 100-fold higher than HaFatB in sunflower developing seeds. This data, together with the high catalytic efficiency of HaFatA, suggested that this enzyme was probably the main contributor to the export of fatty acids and so of oleic acid. Thus, the low specificity of HaFatA towards stearate could be an additional constraint to the accumulation of this acid in sunflower oil.
The structure of the active site of thioesterases has been modeled by analogy with the esterase TEI from *E. coli*. The active site of these enzymes is a hot-dog domain in which those amino acids determining the interaction with the substrate are located [37-39]. In this regard, a study based on site-directed mutagenesis of different residues located in *HaFatA* was carried out to try to produce a new allele with higher activity and/or specificity towards stearoyl-ACP [40]. Thus, several amino acids of the active site were substituted with the bulky and hydrophobic amino acid tryptophan. After several *HaFatA* alleles were assayed, the mutant containing the change Q215W showed catalytic efficiencies that were 2.4-fold higher than those in the wild type *HaFatA*. The mutation did not significantly change the $K_m$ values and the selectivity profile remained unaltered. The effect of expression of this improved allele in different plant tissues was studied by expression in tobacco leaves and Arabidopsis seeds. In addition, the effect of different alleles of *HaFatA* in tobacco leaves was studied by transient expression of these genes. The most remarkable effect of the transformation was an important increase in the TAG content of the leaves, which was 2-fold higher when they were transformed with *HaFatA* from wild type and 4-fold higher when the construct contained the Q215W allele (Fig. 3A). Furthermore, the fatty acid composition of the leaf TAGs was significantly altered, displaying 50% more palmitate and almost double the amount of stearate than control plants expressing the empty vector (Fig. 3B). The increase of saturated fatty acids took place mainly at the expense of the linoleate and linolenate contents of TAG. This result showed that the expression of very active thioesterases in leaves induced a significant movement of fatty acids from the chloroplast that would then be diverted into TAG synthesis presumably to avoid any possible toxic effects. In turn, this caused a notable increase of storage lipids in leaves. Moreover, the increase of the thioesterase activity also increased the export of saturated fatty acids such as stearate, demonstrating that it is possible to increase the flux of carbon into stearic acid by diverting it away from plastid SAD activity. Moreover, this result could be of interest to promoting accumulation of TAGs in vegetative tissues, and, therefore, making an important contribution to the potential energy accumulated in the plant biomass. When these constructs were expressed in Arabidopsis seeds they also caused an increase in saturated fatty acids, although this
did not affect their oil content. The above data showed that it was possible to increase the content of stearate in sunflower mutants by manipulating thioesterase activity.

3. Activation of fatty acids

3.1 Long chain acyl-CoA synthetases

Once fatty acids have been hydrolyzed from the acyl-ACP derivatives they are transported out of the plastid membranes. The mechanism of transport is not well known, but they accumulate in the cytosol, as their corresponding acyl-CoAs. The mechanism of activation is quite efficient so that unesterified fatty acids are normally only present in trace amounts in the cytosol. The activation of unesterified fatty acids takes place by the action of the long chain acyl-CoA synthetases (LACS; [41]). The acyl-CoAs synthesized by these enzymes take part as acyl donors in many reactions of plant metabolism including either synthesis (e.g. TAGs, membrane glycerol- or sphingolipids, surface lipids) or degradation (e.g. β-oxidation).

The first LACS enzyme studied in detail was that of isolated chloroplast envelopes [42]. Nine LACS genes are present in Arabidopsis, encoding proteins with different locations and functions [43]. So, the forms AtLACS 5 and 6 are involved in the glyoxysomal β-oxidation in seeds [44], AtLACS 1 and 2 in the synthesis of surface lipids [45, 46], and AtLACS 9 and AtLACS 1 in the synthesis of seed TAGs [47]. The sequences of the LACS genes from Arabidopsis were used to find consensus sequences that were used to design oligonucleotides in order to clone sunflower LACSs expressed in developing seeds by PCR techniques. In so doing, two LACS genes were isolated from sunflower seed cDNA and were named HaLACS 1 and HaLACS 2 [48]. These genes clustered together with other LACS from dicotyledonous plants in the phylogenetic tree shown in Fig. 4, and were very close to the AtLACS 9 and AtLACS 8 Arabidopsis LACS forms.

The HaLACS 1 and HaLACS 2 were characterized in order to define their role in the synthesis of TAGs in sunflower seeds. The genes did not display any peptide domain that could be related to their subcellular location, as public servers failed to predict any for them. However, the ARAMENOM database did predict the presence of a
hydrophobic domain probably involved in the anchoring of the enzyme to cell membranes. The location of these sunflower LACSSs was studied by making constructs to produce fluorescence tagged HaLACS 1 and HaLACS 2 that were temporarily expressed in N. benthamina cells and observed by confocal microscopy. In this regard, HaLACS 1 was located in chloroplast membranes similarly to AtLACS 9, matching with the Tic-40 marker, although some labeling was present in certain regions of the endoplasmic reticulum (ER) surrounding the chloroplasts (Fig. 5). This latter result could have been caused by artifacts produced by overexpression of the protein within the transformed cells. However the labeling of the ER was present even at very short times after transformation, which indicated a dual location of this enzyme, which fits well with a role in the activation of the fatty acids synthesized de novo in the plastids as well as those later incorporated into glycerolipids in the ER. On the contrary, the form HaLACS 2, which was similar to AtLACS 8, displayed a clear ER location, co-localizing with the concanavalin A marker, just like its Arabidopsis counterpart (Fig. 5).

Both HaLACS forms were overexpressed in E. coli and recovered in the microsomal fraction of the bacteria. It was not possible to purify these enzymes because they did not bind to affinity columns and were not recoverable by solubilization with surfactants like Triton X-100 or Tween-80. These enzymes were assayed with several fatty acid substrates in crude membrane fractions from recombinant bacteria. HaLACS 1 displayed high activity towards monounsaturated oleic and palmitoleic fatty acids. It was also strongly active with polyunsaturated fatty acids (linoleic and α-linolenic acids) but had less activity with palmitic and stearic acids (Fig. 6). Only trace or no activity was found towards very long chain (>18C) fatty acids. The activity profile of the form HaLACS 2 was somewhat different since this isoform displayed its highest activity when it was assayed with palmitic or linoleic acid. The activity was lower towards oleic and α-linolenic acids and, again, stearic acid was a very poor substrate. No activity was recorded for fatty acids above 18C.

The different selectivity profiles of sunflower LACSSs may be due to different roles of these enzymes in lipid metabolism. Thus, HaLACS 1, located in plastid membranes and the ER surrounding them displayed high selectivity towards oleic acid, the main product of the intraplastidial fatty acid synthesis in sunflower, so this would fit well
with a role in the activation of the fatty acids synthesized de novo by plastids. On the other hand, *HaLACS* 2 was located in the ER and was very active with palmitic and linoleic acids, which are the predominant fatty acids in sunflower seed phosphatidylcholine. This would fit well with a role in the acyl turnover that takes place in ER, involving the hydrolysis and reactivation of the fatty acids bound to different phospholipid species, although such a postulate needs more experimental evidence. The low activity of both isoforms towards stearic acid could again be a constraint for the accumulation of this fatty acid in sunflower, and could also contribute to the low rates of oil accumulation in mutant lines accumulating stearic acid in their oils.

Studies of the expression of both genes showed that *HaLACS* 1 was expressed at very high levels in developing sunflower seeds during the whole period of seed development studied, again implicating this enzyme in the process of oil biosynthesis. *HaLACS* 2 was expressed at lower levels in seeds, displaying a maximum of expression at 25 days after anthesis (DAA). This form was also expressed at higher levels in germinating cotyledons and leaves, with expression levels equivalent to its analogous enzyme in root and stems. These results indicated that the regulation of the genes coding for these enzymes was different, which reinforces the hypothesis that they have different functions in sunflower metabolism.

### 3.2 Acyl-CoA pool in developing sunflower seeds.

Studies of the acyl-CoA metabolism in sunflower seeds were completed by measuring the pool size and composition of these derivatives at different stages of development in different mutants [49]. Determination of rates of oil accumulation in different mutants revealed a difference between common sunflower and the high stearic line. The control line displayed a period of quick accumulation of TAGs between 25 and 35 DAA (days after anthesis). The maximum amount of TAGs was reached around the latter time, being close to 50% oil by seed weight. Looking at the acyl-CoA pool during the same period showed that it reached its largest size at the moment of maximum oil synthesis, resulting in a sharp peak (Fig. 7). The pool composition of acyl-CoAs differed
considerably from the fatty acid composition of the seed (Fig. 8). Thus, oleoyl-CoA was the most abundant derivative in contrast with the higher amounts of linoleic acid accumulated in the seed oil. This result showed that the biosynthetic mechanism of sunflower was very efficient at synthesizing and accumulating linoleic acid, a fatty acid which was synthesized in the endoplasmic reticulum from oleic acid and was later quickly channeled to TAGs, resulting in a very efficient process that enabled the rapid oil filling of the seed. On the contrary, the curve of oil accumulation observed in the high stearic CAS-3 mutant was very different. The TAGs were accumulated slowly within a longer period, in a much more gradual way [50]. At the end of the period of study the total content of TAGs was much lower than that in control sunflower. These slower rates of TAG accumulation were also reflected in the curve of acyl-CoA content of this mutant, which was less pronounced than that found for the control line. The acyl-CoA composition in developing high stearic mutants also differed from the acyl composition of the seed, showing a much higher proportion of stearoyl derivatives than expected from the oil composition. This result showed that there was possibly a bottleneck in stearoyl-CoA metabolism in sunflower. Thus, stearate accumulated in the acyl-CoA pool because its incorporation into TAGs was not as efficiently channeled as was linoleic acid in the control line. The reasons for this inefficient metabolism of stearic acid can be ascribed to the selectivity of enzymes responsible for glycerolipid synthesis in the endoplasmic reticulum. Such enzymes use most of the acyl-CoA intermediates coming from plastidial de novo synthesis and the acyl editing reactions involving ER phosphatidylcholine (PC).

4. Assembly of glycerolipids

4.1 Glycerol 3-phosphate:acyl-CoA acyltransferases

The main pathway metabolizing acyl-CoAs in sunflower developing seeds is the Kennedy pathway that is located in the ER [51]. This pathway consists of successive acylations of glycerol 3-phosphate (G3P) to yield phosphatidic acid (PA), which is dephosphorylated to produce a diacylglycerol (DAG). The glycerolipids PA and DAG are the main precursors of the most prevalent phospholipid species like PC,
phosphatidylethanolamine and phosphatidylinositol. The DAGs are also the precursors of TAGs, through a final acylation reaction catalyzed by the enzyme diacylglycerol : acyl-CoA acyltransferase (DGAT), as well as the glycosylglycerides.

The acylation of the G3P starts with the esterification of a fatty acid in its sn-1 position catalyzed by the enzyme glycerol 3-phosphate: acyl-CoA acyltransferase (GPAT). This is a membrane-bound enzyme located in the ER, which has been assayed and characterized in microsomes from developing sunflower seeds [52]. Microsomes from sunflower were active at acylating G3P with a variety of acyl-CoA substrates, displaying higher activities towards linoleoyl-CoA and palmitoyl-CoA. The activity towards oleoyl-CoA was lower, whereas the activity towards stearoyl-CoA was hardly detectable (Fig. 9). Moreover, acyl-CoAs exerted strong product inhibition on GPAT activity, which was markedly decreased at substrate concentrations higher than 0.2-0.3mM. This profile of selectivity fitted well with results from studies on the acyl-CoA pools in different mutants from sunflower described in section 3.2 of this review. Thus, GPAT would contribute to the high flux of incorporation of linoleate into glycerolipids, while its selectivity virtually blocks the incorporation of stearate into the position sn-1 of sunflower seed glycerolipids, thus increasing the accumulation of stearoyl-CoA which is found in the high stearic mutant. This fact causes a high degree of asymmetry in the acyl groups of TAGs from different sunflower mutants [53]. Thus, in Table 3 are shown the expected TAG composition assuming a symmetrical distribution of saturated fatty acids and the actual composition of different mutants. In all cases the level of diunsaturated TAGs (SUU) were higher than predicted at the expense of trisaturated (SSS) and disaturated (SUS) species. This effect was even more important in mutants having a high oleic background, which indicated that the composition of the acyl-CoA pool also has an effect on the selectivity of GPAT in vivo [54].

The gene encoding the GPAT enzyme in plants remained unidentified for a long time [55]. However, Cao et al. [56] identified a group of microsomal enzymes connected to the synthesis of TAGs in mammals. These enzymes displayed similarities with other acyltransferases that were identified as GPATs. The ectopic expression of such enzymes in mammals gave rise to N-ethylmaleimide-sensitive GPAT activity and caused an increase of the accumulated TAGs but not other lipids or other acyltransferase
activities. These enzymes had homologues in Arabidopsis, resulting in some 9 putative genes identified in the genome from this plant, of which 5 displayed acyltransferase activity when expressed in yeast. AtGPAT1 was located in mitochondria and displayed an important role in pollen fertility [57]. Furthermore, AtGPAT4, AtGPAT5 and AtGPAT8 were involved in surface lipid synthesis [58, 59], whereas AtGPAT9 was located in the endoplasmic reticulum and could play a role in TAG biosynthesis [60].

The consensus sequence displayed by different Arabidopsis GPATs has been used to clone two GPATs from sunflower that are located in the endoplasmic reticulum and are expressed in developing seeds. The role of these GPATs on TAG biosynthesis in sunflower is being investigated in this moment.

4.2 Lysophosphatidic acid:acyl-CoA acyltransferase

Sunflower oil (like most vegetable oils) is characterized by having a very low proportion of saturated fatty acids at the sn-2 position of their TAGs. This also occurs in high stearic mutants. Saturated fatty acids are mostly excluded from the sn-2 position of TAGs and individual seeds containing up to 40 % saturated fatty acids in their oil only contained about 2 % of saturated fatty acids at the sn-2 position of their TAGs (Fig. 10). Therefore, most of the TAG species that accumulated were of SUU or SUS types, with very little of SSS. The exclusion of saturated fatty acids from position sn-2 is caused by the substrate selectivity of the enzyme lysophosphatidic acid :acyl-CoA acyltransferase (LPAT). This enzyme is the second in the series of sequential acylations in the TAG biosynthetic pathway that take place in the seed’s endoplasmic reticulum [51]. LPAT has been characterized in crude microsomes from developing sunflower seeds and it shows high activity towards oleoyl-CoA. Some activity was also found for saturated derivatives, palmitoyl-CoA and stearoyl-CoA, but the enzyme displayed very strong substrate inhibition at submillimolar concentrations of these substrates, which may account for the poor incorporation of saturated fatty acids into the sn-2 position of sunflower glycerolipids.

The sequences from several plant LPATs indicated that this enzyme possessed 4 transmembrane domains that anchored it very strongly in the ER membranes [61]. The carboxyl and amino terminals were orientated to the cytosol, as was the catalytic
center where the acylation reaction takes place. Five LPAT genes are present in the Arabidopsis genome [62]. All of them were located in the ER. In sunflower 2 genes coding for LPATs have been sequenced. Both of them were expressed in developing seeds, and they had sequence analogies to AtLPAT2. Studies on the expression of these enzymes showed that they were ubiquitously expressed in sunflower tissues. In seeds they displayed very high levels of expression at the beginning of seed development which decreased abruptly after 15 DAA. This profile of expression was different from those reported for enzymes involved in fatty acid synthesis in sunflower, which peaked at the middle of the period of oil accumulation, coincident with the period of maximum lipid synthesis.

4.3 Diacylglycerol:acyl-CoA acyltransferase

The product of LPAT is phosphatidate, which, in seeds, is quickly dephosphorylated by a phosphatidate phosphatase to diacylglycerol, which is the substrate for the last enzyme of this pathway the diacylglycerol:acyl-CoA acyltransferase (DGAT). This enzyme acylates the last position of the glycerol backbone to produce TAG [63]. In developing seeds TAG accumulates in structures known as oleosomes, consisting of small drops of oil surrounded by a monolayer of phospholipid. The outer surface contains proteins named oleosins, which confer stability to the oil droplets and prevents their coalescence [64].

Two types of DGAT enzymes have been found generally in plants and are named DGAT1 and DGAT2 [55]. They are structurally different and display somewhat different roles within plant lipid metabolism. DGAT1 contains as many as 9 transmembrane domains. It is expressed at high levels in common oilseeds like soybean and rapeseed and is responsible of most of the TAGs synthesized in these species [65-67]. In contrast, DGAT2 is expressed at higher levels in seeds such as castor and tung [68, 69]. Therefore, this isoform has been suggested to play an important role in the accumulation of unusual fatty acids in such seeds. This was confirmed when the DGAT2 from castor was overexpressed in Arabidopsis plants containing the castor hydroxylase, where it caused a 3- to 4-fold increase in the accumulation of ricinoleic acid in the seeds [70].
In sunflower 3 forms of DGAT have been identified and sequenced. Two of them were of the type 1 and one of the type 2. All three genes were expressed in developing sunflower seeds but, unexpectedly, DGAT2 was the isoform displaying the highest level of expression which suggested that, contrary to other species, sunflower accumulated common fatty acids at high levels but with an important contribution of DGAT2. The substrate selectivity of sunflower DGATs have not been yet investigated. However, Wiberg et al. [71] characterized the DGAT activity in microsomes from developing sunflower seeds. In this case, sunflower microsomes were able to incorporate stearoyl-CoA into TAGs at a fair rate, which indicated that this enzyme is responsible for most of the stearic acid incorporated into sunflower TAGs. Thus, the substrate specificity of the different ER acyltransferases accounts for the asymmetric distribution of saturated fatty acids in different sunflower TAGs.

5. Conclusions

Sunflower mutants rich in saturated fatty acids offer a potentially important healthy alternative to hydrogenated and tropical fats. They also represent a non-GMO crop that can be grown in temperate countries. The high content of stearate in different sunflower mutants is caused by a mutation in the SAD enzyme, responsible for the desaturation of stearic acid to oleic acid in the seed plastids. This mutation increased the content in stearic acid in sunflower several fold. However, attempts to produce hybrid lines with contents of stearic acid similar to certain tropical species, like shea or sal, failed because the levels to which stearic acid could be accumulated in sunflower seemed to be limited in some way. The background of the mutant is also essential and is an important factor at determining the maximum proportion of stearic acid that can be accumulated in the seed. Thus, mutants with a high linoleic background accumulated more stearate than mutants rich in oleic acid. In addition, mutants with higher linoleate acid are able to produce a higher proportion of symmetrical TAGs, with a more even distribution of stearic acid between positions sn-1 and 3. The reasons for this are not yet well understood and could involve an up-regulation of stearate desaturase activity in high oleic seeds as well as a preference for substrates carrying esterified linoleate by the acyltransferases in the sunflower ER. From these considerations, biochemical and molecular studies on sunflower showed that the
accumulation of stearate in sunflower is constrained at several levels. Thus, sunflower FatA displayed very low activity towards stearoyl-ACP, and the levels of FatB expression in sunflower are much lower than FatA. Moreover, once stearic acid is exported out of the plastids it is activated at lower rates than other fatty acids because sunflower LACSs display low activity towards stearic acid. Even so stearoyl-CoA is accumulated in the acyl-CoA pools of high stearate mutants, which means that there are also restrictions in the assimilation of the stearoyl-CoA in sunflower. This regulation is due to the properties of the acyltransferases. Thus the incorporation of stearoyl-CoA to the sn-1 position of glycerol is minimal due to substrate inhibition of GPAT by saturated acyl-CoAs. Moreover, saturated fatty acids are usually excluded from the sn-2 position in most oil seeds, due to the substrate selectivity of LPAT. In sunflower the situation is the same and, therefore, sunflower mutants displayed very low amounts of trisaturated TAGs. However, the majority of disaturated TAGs displayed the structure saturated-unsaturated-saturated, which is appropriate for fractionation and for many technological applications. Furthermore, most of the incorporation of stearic acid into TAGs in sunflower takes place through the action of sunflower DGATs, which are less limited in their selectivity towards this fatty acid than GPAT. The different restrictions in the metabolism of stearic acid that take place at different levels of the overall biosynthetic pathway limits the amounts of stearic acid that can be accumulated in sunflower. Such restrictions seem to be enhanced in lines with a high oleate background. These controls in biosynthesis affected both the proportion of stearic acid that can be accumulated and the final oil content of the seed, which is lower in stearate-enriched mutants. This limits the large scale production and exploitation of such oils at the present. Therefore, future developments in this field are being focused on the search for mutants able to better accommodate higher proportions of stearate in their TAGs. Studies include both breeding and mutagenesis. The present-day knowledge that we have of sunflower lipid metabolism should also permit the future improvement of sunflower by genetic engineering. Thus, the improved allele developed from HaFatA would make possible increased plastidial export of stearate, which has been demonstrated to increase the levels of the incorporation of this fatty acid in model plants. Moreover, raised incorporation of stearate onto the sn-1 position of sunflower TAGs would require the
transformation of the crop with an appropriated GPAT gene. The GPAT1 from sunflower seemed to be the isoform mainly involved in TAG biosynthesis. Increasing this activity by its overexpression in sunflower could increase the incorporation of stearate into the sn-1 position. However, in vitro determination of this activity pointed to a strong effect of substrate inhibition by stearoyl-CoA. The high proportion of stearoyl-CoA in the acyl-CoA pool of high stearic lines would thus cause inhibition of GPAT1 and so decrease the metabolic flow into TAGs. All these considerations suggested that transformation using an exogenous GPAT with high specificity towards stearoyl-CoA would be the most effective way of increasing the levels of stearate in sunflower. However, the lack of an effective protocol of sunflower transformation is yet another major impediment for the production of ultra-high stearic sunflower lines.

It will be clear from the above discussion, that the main problems to developing high stearate sunflower have been identified. They have now to be overcome in order to produce a useful new type of oil crop - of utility for both industrial and nutritional purposes.

Acknowledgements

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**Tables**

Table 1. Typical composition of the oils from common sunflower and some sunflower mutants.

<table>
<thead>
<tr>
<th>Fatty acid % (w/w)</th>
<th>Common</th>
<th>High oleic</th>
<th>High stearic</th>
<th>High palmitic</th>
<th>Ultra high oleic</th>
<th>High stearic high oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>3-5</td>
<td>5</td>
<td>3</td>
<td>26</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>18:0</td>
<td>2-3</td>
<td>3</td>
<td>30</td>
<td>2</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>18:1</td>
<td>30-50</td>
<td>75</td>
<td>14</td>
<td>20</td>
<td>91</td>
<td>71</td>
</tr>
<tr>
<td>18:2</td>
<td>40-60</td>
<td>15</td>
<td>50</td>
<td>51</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>20:0</td>
<td>0.5-1</td>
<td>0.5</td>
<td>1</td>
<td>0.4</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>22:0</td>
<td>0.5-1.5</td>
<td>0.8</td>
<td>1.5</td>
<td>0.6</td>
<td>0.6</td>
<td>2</td>
</tr>
<tr>
<td>others</td>
<td>&lt;0.5</td>
<td>&lt;1</td>
<td>&lt;0.5</td>
<td>5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Production: Chemical mutagenesis; Chemical mutagenesis; X-ray irradiation; Breeding; Breeding.

16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:0, arachidic acid; 22:0, behenic acid. Chemical mutagenesis for high oleic line was induced by exposition to dimethyl sulphonate. For high stearic lines the mutagenizing agent was ethylmethane sulphonate. The high palmitic mutant was isolated from a population of seeds mutagenized by irradiation with X-rays.
Table 2. Kinetic parameters of FatA thioesterases from different species toward 18:1-ACP.

<table>
<thead>
<tr>
<th>Thioesterase</th>
<th>Km (µM)</th>
<th>kcat (s(^{-1}))</th>
<th>Kcat/Km (s(^{-1}) µM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castor FatA</td>
<td>0.4</td>
<td>8.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Arabidopsis FatA</td>
<td>3.1</td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Macadamia FatA</td>
<td>0.1</td>
<td>1.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Sunflower FatA WT</td>
<td>0.9</td>
<td>35.6</td>
<td>39.1</td>
</tr>
<tr>
<td>Allele Q215W</td>
<td>2.2</td>
<td>85.4</td>
<td>39.3</td>
</tr>
</tbody>
</table>

See [35, 37, 40, 72]
Table 3. Expected and observed distribution of triacylglycerol species in oils from different sunflower mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Expected*</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[S]</td>
<td>SSS</td>
</tr>
<tr>
<td>High stearic</td>
<td>37</td>
<td>0.7</td>
</tr>
<tr>
<td>High oleic</td>
<td>8.3</td>
<td>0.0</td>
</tr>
<tr>
<td>High stearic-high oleic</td>
<td>33</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Expected for a distribution 1.3-random-2-random as proposed by Van der Wal [54]. [S], total saturated fatty acid content; SSS, trisaturated TAG; SUS, disaturated TAG; SUU, diunsaturated TAG; and UUU, triunsaturated TAG.
Legends to Figures

Fig. 1. Representation of stearic acid versus oleic and linoleic acid contents in the F3 seed generation obtained from F2 segregant plants resulting from crosses of high stearic sunflower with different oleic/linoleic backgrounds. Points represent the composition of individual seeds from two different lines (1 and 2). The contents of fatty acids other than oleic, linoleic or stearic were virtually the same in all the seeds of a given line. Higher contents of stearic acid were observed in the clusters of seeds containing higher levels of linoleic acid (clusters B), than in seeds with high levels of oleic acid (clusters A).

Fig. 2. Phylogenetic comparison of acyl-ACP thioesterases from sunflower with homologous proteins from other species. Plant species included in the phylogenetic tree are: At, Arabidopsis thaliana; Ah, Arachis hypogaea; Bs, Brachypodium sylvaticum; Bj, Brassica juncea; Bn, Brassica napus; Br, Brassica rapa; Ca, Capsicum annuum; Cch, Capsicum chinense; Ct, Carthamus tinctorius; Cca, Cinnamomum camphora; Cs, Coriandrum sativum; Cc, Cuphea calophylla; Ch, Cuphea hookeriana; Cl, Cuphea lanceolata; Cp, Cuphea palustris; Cw, Cuphea wrightii; Db, Diploknema butyracea; Eg, Elaeis guineensis; Gma, Garcinia mangostana; Gm, Glycine max; Gh, Gossypium hirsutum; Ha, Helianthus annuus; Ig, Iris germanica; It, Iris tectorum; Jc, Jatropha curcas; Ml, Madhuca longifolia; Mf, Myristica fragans; Os, Oryza sativa; Pf, Perilla frutescens; Pp, Physcomitrella patens; Ps, Picea sitchensis; Pt, Populus tomentosa; Ptr, Populus trichocarpa; PtrxPd, Populus trichocarpa x Populus deltoids; Rc, Ricinus communis; Ta, Triticum aestivum; Uc, Umbellularia californica; Vv, Vitis vinifera; Zm, Zea mays. Desulfovibrio desulfuricans thioesterase (DdTE, YP_387830.1) was used as outgroup to root the tree. Bootstrap confidence values are indicated.

Fig. 3. Effect of the transient expression of different alleles of sunflower acyl-ACP thioesterase A (FatA) in tobacco leaves. (A) Total triacylglycerol content of leaves. (B) Content of palmitic and stearic acid in the triacylglycerol fraction. T182W corresponded to an inactive allele, FatA WT corresponded to the unmodified sunflower FatA gene and Q215W corresponded to the improved allele.
Fig. 4. Phylogenetic comparison of long chain acyl-CoA synthetases from sunflower with homologous proteins from other species. These species were: Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Ha, Helianthus annuus; Ol, Ostreococcus lucimarinus; Os, Oryza sativa; Pp, Physcomitrella patens; Pt, Populus trichocarpa; Rc, Ricinus communis; Sm, Selaginella moellendorffii; Vv, Vitis vinifera; Zm, Zea mays.

Fig. 5. Studies of localization of long chain acyl-CoA synthetases from sunflower (HaLACS1 and HaLACS2). The genes coding the LACS enzymes were fused with the gene of the green fluorescent protein (GFP) and transiently expressed in tobacco cells. Control proteins were also co-expressed to label the cytosol (red fluorescent protein; RFP), the chloroplastic membranes (TIC40-RFP) and the endoplasmic reticulum (concavaline A-RFP; ConA-RFP). The distribution of these proteins was monitored by confocal microscopy. Column A corresponded to LACS1 and LACS2 labelled with GFP. Column B corresponded to the location of different control proteins labelled with RFP to the RFP itself (row 1). Column C corresponded to the fusion of columns A and B.

Fig. 6. Substrate specificity of long chain acyl-CoA synthetases from sunflower. A: plastidial LACS1, B: LACS2, located in the endoplasmic reticulum. Results corresponded to triplicate assays carried out using membranes from E. coli overexpressing these enzymes. Means ± S.D. shown.

Fig. 7. Total acyl-CoA levels in developing seeds of CAS-6 control sunflower (■) and CAS-3 high stearic sunflower (○) at different stages of development. Means ± S.D.(n=3) shown.

Fig. 8. Composition of acyl-CoA pool (A) and acyl composition of triacylglycerols (B) in developing seeds of CAS-6 control sunflower (black bars) and CAS-3 high stearic sunflower (grey bars) at 25 days after anthesis. Abbreviations as in Table 1 and; 18:3, alpha-linolenic acid; 20:1, gondoic acid; 24:0, lignoceric acid; 26:0, montanic acid. Means ± S.D.(n=3) shown.

Fig. 9. Glycerol 3-phosphate acyltransferase (GPAT) activity in sunflower microsomes. Effect of the acyl donor. Assays were carried out at different concentrations of
stearoyl-CoA (\(\text{-}O\text{-}\)), oleoyl-CoA (\(\text{-}\triangle\text{-}\)) and linoleoyl-CoA (\(\text{-}\nabla\text{-}\)). Means ± S.D. (n=3) shown.

Fig. 10. Distribution of saturated (\(\text{-}\Box\text{-}\)) and unsaturated fatty acids (\(\text{-}\triangledown\text{-}\)) in the sn-2 position of triacylglycerols from sunflower seeds with different contents of saturated fatty acids.
Fig. 2
Fig. 3

A

Triacylglycerols (ng/g Fw)

<table>
<thead>
<tr>
<th></th>
<th>Empty vector</th>
<th>T182W</th>
<th>FatA WT</th>
<th>Q215W</th>
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<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>200</td>
<td>300</td>
<td>450</td>
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</table>

B

Relative content (%)

<table>
<thead>
<tr>
<th></th>
<th>Empty vector</th>
<th>T182W</th>
<th>FatA WT</th>
<th>Q215W</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>18:0</td>
<td>5</td>
<td>8</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 5
Fig. 6

(A) Enzyme Activity (nKat/mg prot)

(B) Acyl-CoA substrate
Fig. 7

Total acyl-CoAs (nmol/g fresh weight) vs. Days after anthesis.
Fig. 8

A

Acyl-CoA (nmole/g FW)

B

Composition (%)

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

16:0-CoA  18:0-CoA  18:1-CoA  18:2-CoA  20:0-CoA  22:0-CoA  24:0-CoA  26:0-CoA

0 10 20 30 40 50

16:0  18:0  18:1  18:2  20:0  22:0
Fig. 9

GPAT specific activity (nmol·min$^{-1}$·mg$^{-1}$ protein) vs. Acyl-CoA (mM)
Fig. 10

 Unsaturated fatty acid content in TAG (%)

 Saturated fatty acid content in TAG (%)