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Lipid characterization of a wrinkled sunflower mutant

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Abstract

As part of a sunflower mutagenesis program carried out to obtain lines with fatty acid profiles in their oils, the half-palmitic CAS-7 line, with ca. 14% palmitic acid content, was isolated. Attempts to obtain a homozygotic line proved to be futile due to the lack of growth of the seedlings 10–12 days after germination. At this age, the seedlings stop growing, displayed a lack of chlorophyll and poor linolenic acid content, a fatty acid intimately linked to photosynthetic membranes. Accordingly, this line has only been maintained through heterozygotic seeds. Likewise, the cotyledons of seeds from this line with medium levels of palmitic acid present a characteristic wrinkled phenotype. In the oil of these seeds, the triacylglycerol content displayed a reduction of approximately 57% with respect to the control line, although a similar reduction was not observed in the polar lipids. Furthermore this mutant has 40.0% of trilinolein, the higher content found until today in sunflower seeds. These data indicate that the CAS-7 mutant possesses a multiple phenotype having a reduced triacylglycerol seed content, a modified intraplastidial fatty acid synthesis, together with a seedling blocked growth and poor green colour and reduced chloroplast development.

Keywords: *Helianthus annuus*; Fatty acids; Lipid; Mutant; Seed; Wrinkled; Linolenic; Palmitic

1. Introduction

In plants, fatty acid biosynthesis up to oleic acid occurs in the chloroplasts or plastids depending on whether the tissue is green or not (Harwood, 1996). The activity of a type II dissociable fatty acid synthetase (FAS) enzyme complex is responsible for the biosynthesis of this fatty acid. The sequential actions of FASIII and FASI produce the palmitoyl-acyl carrier protein (palmitoyl-ACP), which is then lengthened by the action of the FASII complex to stearoyl-ACP. These three enzymatic FAS complexes only differ in one enzyme, the ß-keto-acyl-ACP synthetase (KAS). Finally, stearoyl-ACP can be desaturated to oleoyl-ACP by stearoyl-ACP desaturase (SAD).

Palmitic, stearic and oleic acids can be exported to the cytoplasm following their hydrolysis due to the action of the acyl-ACP thioesterases. Thus, the interaction between the enzymes responsible for the final activities in the main pathway, FASII and SAD, and the acyl-ACP thioesterases would determine the amount of each fatty acid that is exported to the cytoplasm. Accordingly, these enzymes influence the final fatty acid composition of the oilseed. Once acyl-ACPs are hydrolized, acyl-CoA synthase incorporates acyl molecules to the pool of acyl-CoA (Roughan and Slack, 1982), and oleic acid, once incorporated into phosphatidyl-choline, can be desaturated in the endoplasmic reticulum to linoleic and linolenic acids, by the action of the oleate desaturase and the linoleate desaturase, respectively, and latter on incorporated to the acyl-CoA pool. The resulting acyl-CoAs are substrates for the synthesis of complex lipids, mainly triacylglycerols in seeds or galactolipids in green tissues.

In order to study lipid biosynthesis and its regulation, Arabidopsis mutants in which lipid synthesis is affected have been used. These mutants display modifications in

Abbreviations: ACP, acyl carrier protein; DAS, days after sowing; FAS, fatty acid synthetase; KAS, ß-Keto-acyl-ACP synthase; SAD, stearoyl-ACP desaturase; TAG, triacylglycerol.

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the fatty acid or lipid content of their tissues, or in the synthesis of specific lipids (Gallardo et al., 2001; Lemieux et al., 1990; Routaboul et al., 1999). In certain mutant lines, steps in the main pathways of fatty acid biosynthesis are affected, such as the ß-keto-acyl-ACP synthetase (Wu et al., 1994), stearoyl-ACP desaturase (Lightner et al., 1994), acyl-ACP thioesterases (Bonaventure et al., 2003) or oleate desaturase (Horiguchi et al., 2001). These mutants displayed certain modifications in their fatty acid composition and, in the *wrinkled*1 mutant in particular, the glycolytic pathway is affected and the plants display reduced lipid content and a modified fatty acid composition (Gallardo et al., 2001).

Mutant sunflower (*Helianthus annuus* L.) lines have been described with an increase in palmitic acid (e.g. CAS-5 with a 28% palmitic acid in the seed oil) or stearic acid content (e.g. CAS-3 with a 26% of stearic acid) (Osorio et al., 1995; Fernández-Martínez et al., 1997). The biochemical characterization of high-palmitic sunflower lines demonstrated a reduction in KASII activity and elevated thioesterase activity with respect to palmitoyl-ACP (Martínez-Force et al., 1999). Additionally, there is a higher content of uncommon fatty acids derived from the intraplastidial accumulation of palmitic acid, palmitoleic and asclepic acids in the high-palmitic sunflower lines.

Here we describe the selection and characterization of a mutant sunflower obtained by sodium azide mutagenesis that accumulates palmitic and some palmitoleic acids in the seed lipids. This mutant displays a wrinkled seed phenotype and deficient synthesis of linolenic acid during seedling growth.

2. Results

2.1. Mutant selection

Mutated seeds were germinated and sown in the field, and the first generation plants were self-fertilized. The seeds from these plants were collected to select the mutants and the fatty acid composition of the M_2 generation was analyzed. In one of the capitula analyzed, seeds were identified with palmitic acid content higher than the normal. Indeed, when all the seeds from this plant were analyzed, two classes of seeds could be distinguished based on the percentage of palmitic acid that was found in their oil (Fig. 1): (i) seeds with between 6% and 10% of palmitic acid that are denominated here as low-palmitic; and a second class (ii) with a palmitic acid content between 10% and 20% that we called medium-palmitic. Indeed, in none of the seeds were the values of the high-palmitic CAS-5 and CAS-12 mutant lines reached, these mutants having been selected previously and containing about 30% palmitic acid (Álvarez-Ortega et al., 1997). In the first batch of 271 seeds analyzed, 92 seeds were considered as medium-palmitic and these new mutant seeds were named CAS-7.

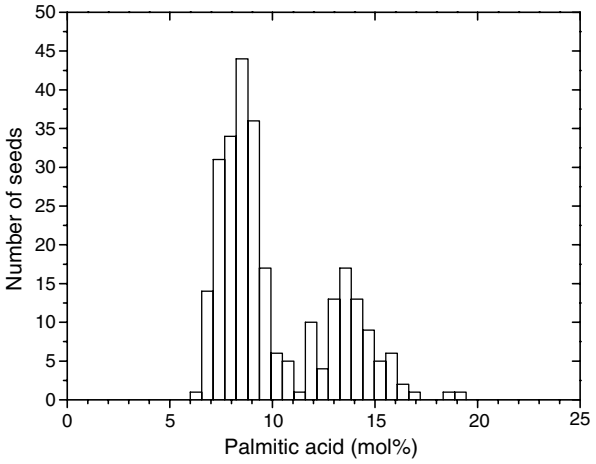

Fig. 1. Histogram of the palmitic acid content in the seeds of the original capitula from which the CAS-7 mutant was identified.

In order to fix the characteristics of this mutant, the seeds with the highest palmitic content were sown. During this selection process it was noted that the seeds with the highest palmitic acid content had problems in germinating and that their growth ceased at 10–12 days after sowing (DAS). More precisely, the cotyledons and hypocotyls of these medium-palmitic seeds remained pale yellow,[1] similar to an etiolated seedling, whilst the seeds with lower palmitic content germinated and grew without any problem. The phenotypic differences associated with the medium-palmitic acid seeds were readily apparent at 5 DAS (Fig. 2A). The three seedlings shown with low-palmitic acid levels displayed a green and healthy vegetative aspect whereas the three seedlings with medium-palmitic acid levels suffered retarded growth and their cotyledons remained pale yellow (below), as was also evident at 12 DAS (Fig. 2B). When the control RHA-274 plants were compared with the medium-palmitic acid plants (Fig. 2C), the control plants are clearly taller and with a greater leaf span. When the germinating CAS-7 mutant seedlings were examined more closely, the lack of normal green colouring and of secondary roots can be more clearly appreciated (Fig. 2D).

During the process of fixation, medium-palmitic acid seeds were only found in some capitula that descended from the subpopulation of low-palmitic seeds that had higher values of palmitic acid. In no case were seeds obtained with values of palmitic acid higher than those observed in the initial M_2 generation.

The fatty acid composition of both classes of seeds, low and medium-palmitic acid, was obtained from various plants of the heterozygotic CAS-7 line during the fixation process (Table 1). As a control, the fatty acids composition of the parental RDF-1-532 and the standard RHA-274 lines are also shown. As can be observed, the increase in

[1] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

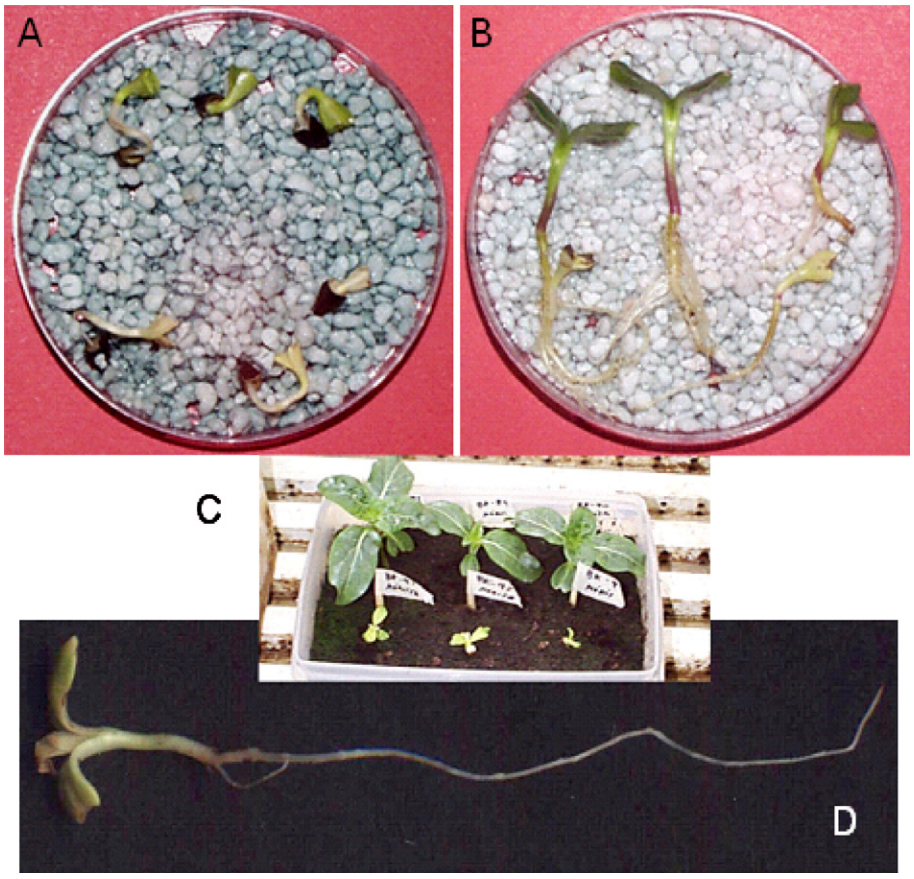

Fig. 2. (A) Seedling of low-palmitic seeds (upper part of the Petri dish) and medium-palmitic (lower part of the dish) 5 days after seeding (DAS); (B) the same seedlings at 12 DAS; (C) photography of the control RHA-274 plants (upper part) and medium-palmitic plants (lower part); and (D) detail of a CAS-7 mutant seedling obtained from medium-palmitic acid seeds.

Table 1
Fatty acid composition of the seed oil from the original mutagenized line RDF-1-532, the control line RHA-274 and from the two different classes of the CAS-7 mutant line defined, low and medium-palmitic

Controls	Fatty acid composition (mol%)[a]					
	16:0	16:1	18:0	18:1	18:1A	18:2
RDF-1-532	7.4. ± 0.6	–	6.8 ± 0.9	25.6 ± 3.7	–	60.2 ± 3.9
RHA-274	6.4 ± 0.5	–	4.9 ± 0.9	30.8 ± 4.0	–	56.2 ± 4.2
CAS-7 Seed type						
Low 16:0	8.6 ± 0.9	0.4 ± 0.8	5.9 ± 1.2	44.3 ± 6.0	0.1 ± 0.4	40.7 ± 6.2
Medium 16:0	14.3 ± 1.9	1.6 ± 1.5	3.3 ± 2.8	11.2 ± 9.9	2.0 ± 1.5	67.5 ± 10.4

The data are expressed as the mean ± standard deviation of the seed collected from plants cultivated during the first three field selection years.
[a] 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:1A, asclepic acid; and 18:2, linoleic acid.

palmitic acid content of the medium-palmitic acid seeds was translated into higher levels of linoleic acid, and in a reduction in stearic and oleic acid levels which could represent a compensatory mechanism to maintain certain membrane fluidity due to the higher palmitic acid content (Table 1). Likewise, there was an accumulation of unusual $n-7$ fatty acids palmitoleic and asclepic ($n-7$ isomer of octadecanoic acid), a similar effect to that observed previously in the high-palmitic CAS-5 line on a linoleic background (Martínez-Force et al., 1999). The accumulation of these fatty acids was related to the uncoupling of the channelling that existed in the intraplastidial synthesis of fatty acids (Martínez-Force and Garcés, 2002).

The normal selection procedure based on the palmitic content, self-fertilization and selection of the seeds for the desired character did not permit the medium-palmitic acid characteristic to be fixed in this line. This could only be maintained in heterozygosis in plants that displayed seeds values of palmitic acid similar to normal plants, and on occasion with values between the normal plants and the medium-palmitic plants (8–12%). In general, the mean palmitic content could not be increased during the fixation

process even though the range of variation was generally fairly wide.

This mutant presents seeds an increased content of palmitic acid, but without reaching the values described in other high-palmitic sunflower mutants (Osorio et al., 1995). Furthermore, this mutant displays higher linoleic acid content than that described to date. Initially, and on the basis of earlier data (Martínez-Force et al., 1999), the high content of palmitic acid and its $n-7$ derivatives (palmitoleic and asclepic acids) suggest a defect or a weaker expression of the gene that encodes the sunflower KASII enzyme.

2.2. Fat content

During the fatty acid analysis of half seeds, the main palmitic acid content, as well as the lack of seedling development, was associated with a non-swollen wrinkled phenotype of the cotyledon in the seed (Fig. 3). Extracting the oil from the seed, the fat content of the seed was quantified in the standard and wrinkled cotyledons (Table 2). Accordingly, the fat content was three times lower with respect to the weight of the achene in the wrinkled seeds with higher palmitic acid content. The distinct lipid classes were separated on TLC plates and quantified, demonstrating that the polar lipid content was similar in both types of seeds, a standard composition in the standard seeds and rich in palmitic acid in rough seeds. In contrast, the triglyceride content of the wrinkled seeds was much lower.

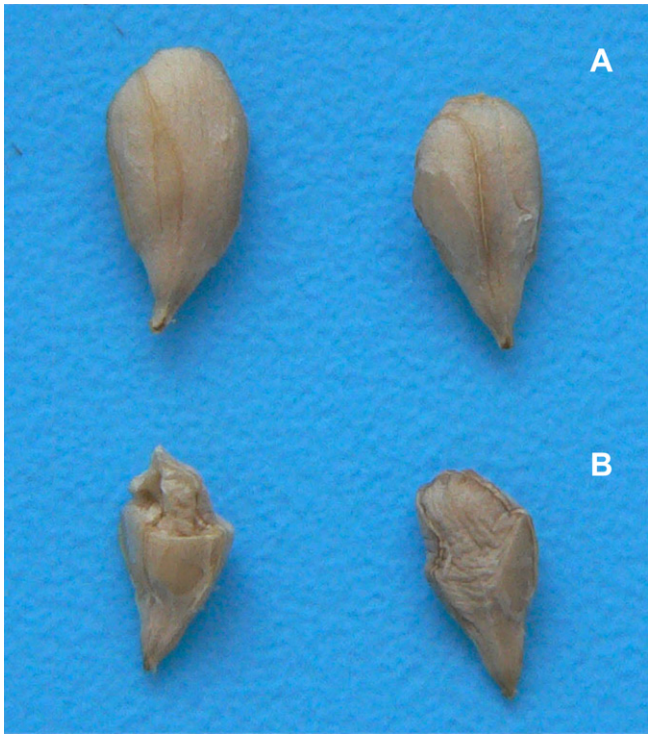

Fig. 3. Segregant seeds of the CAS-7 line. (A) Turgent seeds with normal fatty acid composition; and (B) Wrinkled seed with higher palmitic acid content and its derivatives.

This wrinkled phenotype is usually associated with the lack of some substances that form part of the reserves in the seeds: starch (Bhattacharrya et al., 1990), protein reserves (Focks and Benning, 1998) or oil (Gallardo et al., 2001). The poorer fat content of these wrinkled seeds with a medium palmitic acid phenotype led us to believe that this poor oil content could be responsible for the wrinkled phenotype, and that this could be due to a defect in the regulation of lipid biosynthesis in the developing seeds, that most significantly affect the triacylglycerol and diacylglycerol content, whilst the polar lipid content remains constant.

On the other hand, it has also been shown that in the initial stages of their formation, sunflower seeds predominantly synthesize membrane phospholipids. The seeds in this first stage of development display greater palmitic acid content than in posterior stages (Martínez-Force et al., 1998). Subsequently, between the second and fifth week after flowering, the seeds begin to actively synthesize TAGs, and the polar lipid become a minority component (Triki et al., 1997). All this suggests that the initial stages of seed formation are normal in the CAS-7 mutant, but that when active synthesis of TAGs commences it does so at a low rate.

The blockage or reduction of the growth of the seedlings of wrinkled seeds of the medium-palmitic acid CAS-7 line suggests that the lower TAG content is not the cause of the deficient development but rather that both these two characteristics are linked or they are a manifestations of the same mutation.

2.3. Composition of the triacylglycerol fatty acids in mutant seeds

The TAG fatty acid composition was similar to that of the total lipids although certain differences were evident (Table 3). While TAGs usually constitute ca. 94% of the total seed lipids in standard sunflowers (Garcés et al., 1989), this does not occur in CAS-7 as could be seen above. Hence, it would be logical to suppose that differences might exist between the total fatty acid composition of the TAGs in CAS-7. Accordingly, we found that the fatty acid content of these TAGs was similar in the oil of standard seeds with 6.1% palmitic acid and that of the wrinkled seeds with 11% palmitic acid. These latter seeds also contained a small quantity of palmitoleic acid due to the desaturation of palmitic acid, as occurs to a lesser extent in high-palmitic sunflower lines (Martínez-Force et al., 1999).

As such, the fatty acid composition of polar lipids is more significant in the final oil composition than in normal seeds. The polar lipids in the seeds of standard sunflower lines present a palmitic acid content between 11.5% and 13% (Álvarez-Ortega et al., 1997). This could in part explain why there is medium-palmitic content in these seeds, especially if we also take into consideration the possible deregulation of some of the enzymes of the intraplas-

Table 2
Fat content and fatty acid composition in the distinct types of lipids, triacylglycerol (TAG), diacylglycerol (DAG), polar lipid (PL), free fatty acids (FFA) and waxes (WA) in the oil of the smooth and wrinkled seeds of the CAS-7 mutant

Seed type	% Fat	Lipid type (units)				
		TAG	DAG	PL	FFA	WA
Wrinkled	10.7 ± 2.2	4445	895	1125	490	346
Standard	37.3 ± 4.5	10407	2060	1070	955	356
Ratio wrinkled/standard	0.29	0.43	0.43	1.05	0.51	0.97

The same amount of oil from each line was separated by TLC (see experimental for procedures). The TAG content of DAG, PL, FFA and WA are expressed in arbitrary units.

Table 3
Fatty acid composition of purified triacylglycerol from low and medium-palmitic acid content CAS-7 seeds

Seed type	Fatty acid composition (mol%)							
	16:0	16:1	18:0	18:1	18:1A	18:2	20:0	22:0
Low	6.1 ± 1.3	–	5.7 ± 2.3	28.2 ± 4.3	1.1 ± 0.3	56.4 ± 7.0	0.1 ± 0.1	2.5 ± 0.4
Medium	11.0 ± 1.7	1.2 ± 0.5	3.8 ± 1.7	9.8 ± 3.0	2.7 ± 1.0	69.7 ± 8.4	0.5 ± 0.1	1.3 ± 0.5

The data are expressed as the mean ± standard deviation of the seed collected from three plants.

tidial fatty acid biosynthetic pathway, as occurs in the wrinkled1 Arabidopsis mutant (Ruuska et al., 2002).

2.4. Analysis of the molecular species in the triacylglycerol

The distinctive composition of fatty acids in the mutant sunflower lines is reflected in the profile of the TAG molecular species (Finkelstein and Somerville, 1990; Serrano-Vega et al., 2005). The composition of the distinct TAG species of the low and medium-palmitic segregants of CAS-7 is shown in Table 4. The TAG species serve as a more precise measure than the simple fatty acid composition, enabling the specific characteristics of each sunflower line to be identified. In this case, the most outstanding feature is the high content of TAG species that contain linoleic acid, the main fatty acid in medium-palmitic acid seeds, and of those with a molecule of palmitic acid that in principle might be expected due to the higher content of palmitic and linoleic acid. However, the content of other species in which linoleic acid is accompanied by other types of fatty acids is lower, such as the SOL, OOL and OLL TAGs. It is remarkable that the seed oil of the medium-palmitic acid CAS-7 line presents the highest LLL content described to date in the sunflower, around two-three times that found in the standard lines, and that of high-palmitic mutants and the lines derived from these (Finkelstein and Somerville, 1990; Serrano-Vega et al., 2005). The disaturated PLP and the PLL TAGs are much more abundant in the wrinkled seeds, whereas in the standard seeds, TAGs with one or two molecules of oleic acid such as, OOL and OLL are the main species found.

Table 4
Composition of the molecular species in triacylglycerol from oils of segregant seeds with a normal fatty acid composition and medium-palmitic acid seeds of the CAS-7 mutant sunflower line

TAG	Triacylglycerol composition (mol%)	
	Low-palmitic	Medium-palmitic
PLP	0.7 ± 0.2	3.1 ± 1.7
PPoL	–	1.4 ± 0.5
POO	2.0 ± 0.5	0.4 ± 0.3
PLS	1.0 ± 0.2	1.4 ± 0.3
POL	6.1 ± 1.3	3.3 ± 1.2
PAsL	–	1.6 ± 0.6
PLL	6.8 ± 0.1	19.3 ± 4.4
PoLL	–	1.7 ± 0.4
SOO	1.3 ± 0.2	–
SLS	0.4 ± 0.2	–
OOO	3.8 ± 2.4	–
SOL	4.4 ± 1.6	1.1 ± 0.7
OOL	13.8 ± 3.9	2.3 ± 0.8
SLL	6.8 ± 3.6	4.0 ± 1.9
OLL	27.8 ± 1.3	10.6 ± 4.5
AsLL	–	3.5 ± 2.1
LLL	20.1 ± 2.7	40.0 ± 6.1
SLA	–	0.9 ± 1.3
LLA	–	0.8 ± 0.3
SOB	0.8 ± 0.5	–
OOB	1.6 ± 0.5	–
SLB	0.7 ± 0.1	0.6 ± 0.4
OLB	1.1 ± 0.1	1.6 ± 0.7
LLB	1.0 ± 0.3	3.2 ± 1.2

The data are expressed as the mean ± standard deviation of the seed collected from three plants.

2.5. Fatty acid composition in the vegetative tissues of seedlings 20 days after germination

Both the lack of colour of the plants from the medium-palmitic acid seeds, as well as the blockage in their growth, led us to study the lipid composition of the distinct tissues of these seedlings. The fatty acid composition of the roots, the hypocotyl and the cotyledons of the seedlings was analyzed, both in low as well as medium-palmitic acid CAS-7 plants. The composition of both the low and medium-palmitic acid seeds displayed differences in the fatty acid profiles (Fig. 4), principally in the palmitic acid content.

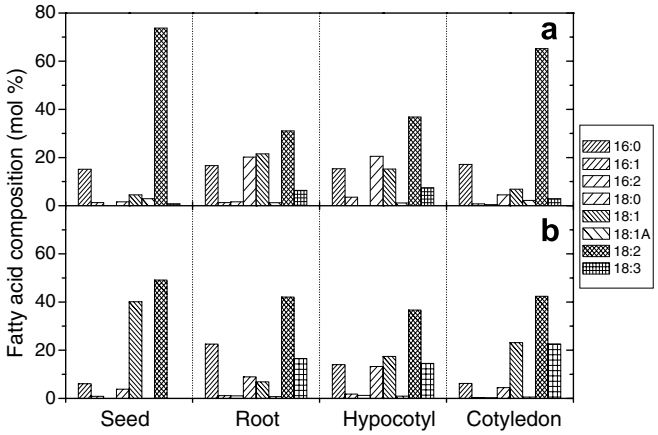

Fig. 4. Composition of fatty acids in the total lipids of mature seeds and in distinct tissues (root, hypocotyl and cotelydon) of 20 day old CAS-7 (a) and normal RDF-1-532 seedlings (b). 16:0, palmitic acid; 16:1, palmitoleic; 16:2, palmitolinoleic; 18:0, stearic; 18:1, oleic; 18:1A, asclepic; 18:2, linoleic; 18:3, linolenic.

Accordingly, these differences influenced the ratio of oleic and linoleic acid in the seed. Once the seeds had germinated and the plants had begun to develop, the differences were much greater due to the linolenic acid content, and its near absence in the cotyledon augmented these differences even further. Together, this indicates that in the seedlings that grew from medium-palmitic acid seeds there was a deficiency in the desaturation of linoleic acid to linolenic acid. In normal sunflower seedlings, as well as in the seeds of the low-palmitic CAS-7 line and those of other plant species, the increase in the linolenic acid content is associated with the green colour of the cotyledon and the differentiation of the plastids into chloroplasts. In the case of the seedlings originated from the medium-palmitic seeds of the CAS-7 line, linoleic acid was not desaturated nor did the photosynthetic tissues develop a normal green colour. In Arabidopsis, whilst the synthesis of linolenic acid in seeds is produced in the endoplasmic reticulum by the action of the ω-3 desaturase encoded by the *fad3* gene, its synthesis in green tissues is mainly produced in the plastid and it is carried out by the ω-3 desaturase encoded by the *fad7* gene. This indicated that the lack of expression of this gene may be responsible for the lack of linolenic acid in these CAS-7 seedlings. It remains to be determined whether the lack of differentiation occurs because the linoleic acid is not desaturated. However, it seems more likely that the regulatory mechanisms that facilitate the differentiation of the seed plastids into functional chloroplasts fail and that the synthesis of trienoic fatty acids is also controlled within this pathway of differentiation.

3. Conclusions

A sunflower mutant, called CAS-7, with half-palmitic content in the seed has been isolated. It was not possible to fix this mutant, seeds with ca. 14% or above palmitic acid content stop growth at 10–12 days after seeding, displaying a poor linolenic acid content. The CAS-7 cotyledons with medium levels of palmitic acid present a characteristic wrinkled phenotype, having a reduction of TAG and DAG contents, although a similar reduction was not observed in the polar lipids. Together, these data indicate that the new CAS-7 mutant possesses a multiple mutant phenotype; reduced seed TAGs content, increased palmitic acid content in seed lipids, and that the growth of the seedling ceased at 10–12 DAS, having a very low linolenic acid synthesis.

4. Experimental

4.1. Plant material and growth conditions

The lines used in this study were the parental RDF-1-532, the mutant CAS-7 line, and the public line RHA-274 with a normal fatty composition. Plants were cultivated in the field, during spring and summer, and in growth chambers at 25/15 °C (day/night), with 16-h photoperiod and 150 $\mu mol\ m^{-2}\ s^{-1}$ light intensity. Tissue samples and seeds from three different plants were analyzed in each experiment. Seeds were sown on perlite, and samples of the cotyledons, hypocotyls, stems and roots were taken for up to 20 DAS.

4.2. Sunflower mutant selection

Chemical mutagenesis with sodium azide was carried out, 4 mM in 0.1 M potassium citrate buffer (pH 3) according to the method described in Osorio et al. (1995). Mutagenesis was performed by exposing 3000 seeds to the mutagen for 1 h at room temperature and the mutagenized seeds were sown in the field.. M_1 plants were self-pollinated by covering them with a paper bag and the M_2 seeds were collected for mutant selection. Two seeds were taken from each individual capitulum and their fatty acid composition was determined. The distal portion of the cotyledons was used for lipid analysis (half-seed method) and the rest of the seed containing the embryo was stored. If the half-seed analysis showed a fatty acid composition of interest, the stored part of the seed containing the embryo was germinated and grown to obtain the next seed generation by self-pollination as above.

4.3. Lipids extraction, separation and quantification

Peeled seeds and vegetative tissues were ground in a glass tube with sand and the total lipids were extracted in hexane/2-propanol (Hara and Radin, 1978). To quantify the total oil content, the lipids were weighed after the initial extraction. To quantify the different lipids, the mixture was separated into triacylglycerols (TAGs), diacylglycerol and polarlipids by TLC on silica gel plates, which were developed with hexane/ethyl ether/formic acid (75:25:1, by vol). To detect the lipid spots, TLC plates were charred,

scanned and the different fractions were quantified using the Scion Image program v4.0.2 (Scion Corporation, Frederick, MD).

4.4. Triacylglycerols purification

Total lipids were extracted and separated by TLC as described above. To detect the TAG positions, TLC plates were partially covered with a glass plate and exposed to iodine vapour. Unexposed TAG fractions were scraped off the plates and eluted from silica with hexane/ethyl ether (95:5, v:v). Oil samples from two plants were used for TAG analysis. In each case, samples of purified TAG were analyzed by GLC. The data presented are the average of three measurements.

4.5. Lipid analysis

To determine the fatty acid composition of the seed and tissues samples (roots, hypocotyls/stem and cotyledons), the lipids were extracted, transmethylated and purified by a modified method of the one-step method described by Garcés and Mancha (1993). Half-seed samples were heated to 80 °C for 2 h in MeOH:toluene:dimethoxypropane:H_2SO_4:heptane (33:14:2:1:50, by vol) and after cooling, the fatty acid methyl esters were recovered in the upper phase. Analysis of the fatty acid methyl ester composition was carried out by 6890A gas chromatography (Agilent, Palo Alto, CA) using a SP-2380 capillary column of fused silica (30 m length; 0.25 mm i.d.; 0.20 μm film thickness: Supelco, Bellefonte, PA) quantified by FID. Hydrogen was used as the carrier gas with a linear gas rate of 28 cm/s. The injector and detector temperature was 220 °C and the oven temperature was 170 °C. Samples (1 μl) were injected with a split ratio of 1/50. Fatty acids were identified by comparison to known standards (Sigma, S. Louis, MI), except for asclepic acid that has been identify by comparison with mutant CAS-5 oil (Martínez-Force et al., 1999).

TAG species were separated by GLC and quantified by FID using a Agilent 6890 gas chromatograph with a Quadrex aluminium-clad bonded methyl 65% phenyl silicone 15 M, 400-65HT-15-0.1F column (New Haven, CT). The TAGs were identified and the data corrected for the relative response of the FID (Fernandez-Moya et al., 2000). After a 5 min holding period, the oven temperature was ramped from 340 to 355 °C at 1 °C/min, the detector and injector temperatures were 400 °C, and the split ratio was 1:100. Hydrogen was used as the carrier gas at a linear rate of 31 cm/s.

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References

Álvarez-Ortega, R., Cantisán, S., Martínez-Force, E., Garcés, R., 1997. Characterization of polar and nonpolar seed lipid classes from highly saturated fatty acid sunflower mutants. Lipids 32, 833–837.

Bhattacharrya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C., Martin, C., 1990. The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell 60, 115–122.

Bonaventure, G., Salas, J.J., Pollard, M.R., Ohlrogge, J.B., 2003. Disruption of the FATB gene in Arabidopsis demonstrates an essential role of saturated fatty acids in plant growth. Plant Cell 15, 1020–1033.

Fernández-Martínez, J.M., Mancha, M., Osorio, J., Garcés, R., 1997. Sunflower mutant containing high levels of palmitic acid in high oleic backgrounds. Euphytica 97, 113–116.

Fernandez-Moya, V., Martinez-Force, E., Garces, R., 2000. Identification of triacylglycerol species from high-saturated sunflower (*Helianthus annuus*) mutants. J. Agric. Food Chem. 48, 764–769.

Finkelstein, R.R., Somerville, C.R., 1990. Three classes of abscisic (ABA)-insensitive mutations of Arabidopsis define genes that control overlapping subsets of ABA responses. Plant Physiol. 94, 1171–1179.

Focks, N., Benning, C., 1998. Wrinkled1: a novel, low-seed-oil mutant of *Arabidopsis* with a deficiency in the seed-specific regulation of carbohydrate metabolism. Plant Physiol. 118, 91–101.

Gallardo, K., Job, C., Groot, S.P.C., Puype, M., Demol, H., Vandekerckhove, J., Job, D., 2001. Proteomic analysis of Arabidopsis seed germination and priming. Plant Physiol. 126, 835–848.

Garcés, R., Mancha, M., 1993. One-step lipid extraction and fatty-acid methyl-esters preparation from fresh plant-tissues. Anal. Biochem. 211, 139–143.

Garcés, R., García, J.M., Mancha, M., 1989. Lipid characterization in seeds of a high oleic acid sunflower mutant. Phytochemistry 28, 2597–2600.

Hara, A., Radin, N.S., 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90, 420–426.

Harwood, J.L., 1996. Recent advances in the biosynthesis of plant fatty acids. Biochim. Biophys. Acta 1301, 7–56.

Horiguchi, G., Kodama, H., Iba, K., 2001. Characterization of Arabidopsis mutants that are associated with altered C18 unsaturated fatty acid metabolism. Plant Sci. 161, 1117–1123.

Lemieux, B., Miquel, M., Somerville, C., Browse, J., 1990. Mutants of *Arabidopsis* with alterations in seed lipid fatty-acid composition. Theor. Appl. Genet. 80, 234–240.

Lightner, J., Wu, J.R., Browse, J., 1994. A mutant of *Arabidopsis* with increased levels of stearic-acid. Plant Physiol. 106, 1443–1451.

Martínez-Force, E., Garcés, R., 2002. Dynamic channelling during de novo fatty acid biosynthesis in *Helianthus annuus* seeds. Plant Physiol. Biochem. 40, 383–391.

Martínez-Force, E., Álvarez-Ortega, R., Cantisán, S., Garcés, R., 1998. Fatty acid composition in developing high saturated sunflower (*Helianthus annuus*) seeds: maturation changes and temperature effect. J. Agric. Food Chem. 46, 3577–3582.

Martínez-Force, E., Álvarez-Ortega, R., Garcés, R., 1999. Enzymatic characterisation of high-palmitic acid sunflower (*Helianthus annuus* L.) mutants. Planta 207, 533–538.

Osorio, J., Fernández-Martínez, J., Mancha, M., Garcés, R., 1995. Mutant sunflowers with high-concentration of saturated fatty-acids in the oil. Crop Sci. 35, 739–742.

Roughan, P.G., Slack, C.R., 1982. Cellular-organization of glycerolipid metabolism. Ann. Rev. Plant Physiol. Plant Mol. Biol. 33, 97–132.

Routaboul, J-M., Benning, C., Bechtold, N., Caboche, M., Lepinies, L., 1999. The TAG1 locus of *Arabidopsis* encodes for a diacylglycerol acyltransferase. Plant Physiol. Biochem. 37, 831–840.

Ruuska, S.A., Girke, T., Benning, C., Ohlrogge, J.B., 2002. Contrapuntal networks of gene expression during Arabidopsis seed filling. Plant Cell 14, 1191–1206.

Serrano-Vega, M.J., Martínez-Force, E., Garcés, R., 2005. Lipid characterization of seed oils from high-palmitic, low-palmitoleic, and very high-stearic acid sunflower lines. Lipids 40, 369–374.

Triki, S., Ben Hamida, J., Mazliak, P., 1997. Study of the metabolism of lipid reserves in ripening sunflower seeds: a tracing experiment with [1-C-14] acetate. OCL-Ol. Corps Gras Lipid. 4, 451–458.

Wu, J.R., James, D.W., Dooner, H.K., Browse, J., 1994. A mutant of Arabidopsis deficient in the elongation of palmitic acid. Plant Physiol. 106, 143–150.