Metabolism of Triacylglycerol Species during Seed Germination in Fatty Acid Sunflower (*Helianthus annuus*) Mutants

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Sunflower mutant lines with high saturated fatty acid content (palmitic or stearic) in the oil have a completely different set of triacylglycerols (TAG), some of which were not found in standard sunflowers. For optimum seed germination, all of these new TAG species must be effectively catabolized. The behavior of the TAG composition during germination in cotyledons of all these mutant lines showed two different phases: an initial phase (between 0 and 2 days after sowing) with a higher catalytic activity and a preference for TAG containing at least two oleic acid molecules and a second phase with lower TAG degradation rate and a low preference for TAG containing two saturated fatty acids usually accompanied by linoleic acid. Despite the elevated content of saturated fatty acids in some TAG species, the total TAG degradation rate and germination process were similar in these lines, suggesting that sunflower seed lipases do not show a marked preference for any TAG species.

Keywords: *Helianthus annuus; germination; saturated fatty acids; seed; sunflower; triacylglycerol*

INTRODUCTION

During the germination of oilseeds, storage lipids, mainly triacylglycerols (TAG), are catabolized and new synthesis of polar lipids (PL), phospholipids, and galactolipids, takes place (Ichihara and Noda, 1980). Most TAG are hydrolyzed in the first 10 days after sowing (DAS) by the action of lipases (Ichihara and Noda, 1980; Wilson and Kwanyuen, 1986). The substrate specificity of plant lipases has been studied in different plant species. Studies performed with corn, castor bean, rapeseed, and elm suggest that a correlation exists between lipase specificity and the fatty acid composition in the seeds (Lin and Huang, 1986), that is, lipases were found to be relatively specific for TAG containing the major fatty acid components of the storage TAG in those species. However, studies carried out with seeds of *Pinus edulis* seemed to indicate that the lipases were unspecific for individual TAG during germination, and levels of all TAG were depleted similarly (Hammer and Murphy, 1994). Other authors have connected the germination in *Dioscorea tokoro* with degradation of specific TAG molecules (Okagami and Terui, 1996). At 20 °C, TAG with one or no linoleic acid (L) combined with palmitic (P), stearic (S), oleic (O), and linolenic (Ln) acids, such as OLO, OOO, PLO, POO, OLS, and OLnO, are catabolized more rapidly than any other TAG. All previous works were carried out in lines (or plant species) with normal TAG composition. Seeds of mutant sunflower lines exhibiting a high-saturated fatty acid phenotype (25−30% palmitic acid; 25−30% stearic acid) (Osorio et al., 1995; Fernández-Martínez et al., 1997) show modifications in their oil quality, mostly TAG (Fernández-Moya et al., 2000). These new oils are a complete set of oils enriched in palmitic, stearic, and/or oleic acids (Álvarez-Ortega et al., 1997), having increased content of these fatty acids and/or oleic acid in all lipid classes. An unusually high proportion of special TAG with one or two saturated fatty acids or oleic acid appears, making this set of mutant lines a good model system to study the TAG catabolism because of the lipase selectivity for different TAG molecules during seed germination. The high proportion of TAG with saturated fatty acids in these seeds might pose a problem when the TAG are catabolized during the growth of the seedlings. High-stearic mutant lines from other species show germination problems in seeds with a content of stearic acid >22% (Rahman et al., 1997). With the aid of these mutants it is now possible to compare the degradation of specific TAG with different fatty acid compositions. In this work the rate of TAG degradation and the behavior of TAG species during the seed germination of mutant and normal sunflower lines were studied.

MATERIALS AND METHODS

Plant Material. Sunflower (*Helianthus annuus* L.) seeds from mutant lines CAS-3, with high stearic acid content, and CAS-5 and CAS-12, with high palmitic acid content, were used in this work (Osorio et al., 1995; Fernández-Martínez et al., 1997). CAS-12 is additionally a high-oleic mutant. The control seeds were from standard lines RHA-274 and CAS-6 and the high-oleic line G-8. Seeds were sown on vermiculite at 25 °C in the dark during 3 days and then transferred to a growth chamber, with a 16 h photoperiod and 300 μmol m^{-2} s^{-1} light intensity. Cotyledon samples were collected at 2, 4, and 6 DAS. Two replicates were made for each experiment, and an independent sample was taken and analyzed for each. Data are the mean of the two experiments, the standard deviation (SD) being <10% of the mean value.

Lipid Extraction and Separation. Seeds were peeled and then ground in a screw-cap glass tube (10 × 13 mm) with a pestle and sand. Total lipids were extracted (Hara and Radin, 1978) and TAG purified in two ways: (i) Thin-layer chroma-

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Table 1. Fatty Acid Composition of TAG (A) and PL (B) of Mutant Lines CAS-3, CAS-5, and CAS-12 and Controls RHA-274 (a Standard Fatty Acid Composition Line) and G-8 (a High-Oleic Line) at 0 and 6 DAS

(A) TAG

		fatty acid composition (mol %)								
	DAS	C16:0	C16:1	C16:2	C18:0	C18:1	C18:1A[b]	C18:2	C20:0	C22:0
RHA-274	0	5.8	–[c]	–	6.2	29.2	–	57.8	–	1.0
	6	5.5	–	–	4.9	32.0	–	56.0	0.8	0.9
G-8	0	3.8	–	–	5.3	88.5	–	1.4	–	1.1
	6	4.8	–	–	4.5	85.4	–	3.6	0.5	1.3
CAS-3	0	5.2	–	–	26.4	11.5	–	54.3	1.4	1.4
	6	6.4	–	–	26.0	12.4	–	51.1	2.0	2.1
CAS-5	0	29.9	4.3	1.0	4.2	4.8	5.4	49.3	–	1.1
	6	27.6	4.0	1.5	3.6	4.9	5.2	50.8	0.6	1.8
CAS-12	0	25.5	6.2	–	2.3	57.4	4.7	2.5	–	1.3
	6	24.2	5.8	–	2.3	57.7	4.2	3.4	1.0	1.3

(B) Polar Lipids

		fatty acid composition (mol %)					
	DAS	C16:0	C16:1	C18:0	C18:1[d]	C18:2	C18:3
RHA-274	0	8.7	–	8.9	24.2	58.2	–
	6	10.3	–	7.9	8.2	43.4	30.2
G-8	0	11.9	–	5.2	79.4	3.4	–
	6	11.5	–	4.1	31.5	29.1	23.7
CAS-3	0	7.2	–	17.3	12.1	63.5	–
	6	10.5	–	16.8	5.6	41.7	25.2
CAS-5	0	27.4	2.1	2.4	6.3	61.8	–
	6	16.0	1.1	7.8	29.5	30.6	15.0
CAS-12	0	16.3	4.5	4.8	67.9	6.1	–
	6	20.7	2.4	6.8	17.5	34.0	17.8

[a] Mean of two independent experiments, SD < 10% of the mean value. [b] C18:1A is the $n-7$ isomer of C18:1, trivial name asclepic acid. [c] <0.5%. [d] Sum of both isomers; oleic (isomer $n-9$) and asclepic (isomer $n-7$) acids.

tography (TLC) silica gel plates, thickness = 0.25 mm, were developed with hexane, ethyl ether, and formic acid (75:25:1, v/v). TLC plates were partially covered with a glass plate and exposed to iodine vapors; unexposed TAG fractions were scraped off the plates and eluted from silica with hexane and ethyl ether (95:5, v/v). (ii) TAG were eluted from silica and Celite (80:20) columns with hexane and ethyl ether (95:5, v/v). Previously, a silica–Celite mixture was activated at 110 °C during 30 min.

Lipid Analysis. Fatty acid methyl esters were obtained from the isolated lipids (Garcés and Mancha, 1993) by heating the samples at 80 °C for 1 h in a 3 mL solution of methanol, toluene, and H_2SO_4 (88:10:2, v/v). After cooling, 1 mL of heptane was added and mixed. The fatty acid methyl esters were recovered from the upper phase and then separated and quantified using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) with a Supelco SP-2380 capillary column (30 m length; 0.32 mm i.d.; 0.20 μm film thickness) of fused silica (Bellefonte, PA). Hydrogen was used as carrier gas. The fatty acids were identified by comparison with known standards.

TAG Analysis. TAG species were separated and quantified by GLC using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA) with a Quadrex aluminum-clad bonded methyl 65% phenyl silicone 15M, 400-65HT-15-0.1F. Detector and injector temperatures were 400 °C, whereas a temperature gradient after 5 min from 340 to 355 °C (1 °C/min) was used in the oven. Hydrogen was used as carrier gas. TAG molecular species were identified and quantified according to the method of Fernández-Moya et al. (2000).

RESULTS

Changes in TAG and PL Fatty Acid Composition during Early Stages of Germination. During sunflower seed germination two lipid classes are mainly found in the cotyledon: TAG, the most abundant lipid in dry seeds, and PL, mostly chloroplastic, that become the principal lipid component after a few days. During germination, as Table 1 shows, only the fatty acid composition of PL changes significantly in all lines, both control and mutant lines. These changes are mainly due to the synthesis of linolenic acid, which is not present in dry seeds but is the main fatty acid in chloroplastic lipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol, etc.). Whereas the oleic acid content decreased in all cases except in the high-palmitic line CAS-5, the linoleic acid (linolenic direct precursor) increased in the two high-oleic lines (G-8 and CAS-12), the ones showing lower linoleic levels in the dry seed, and decreased in the others. With respect to TAG fatty acid composition (Table 1A) only small differences were found, and it was not possible to determine from these data whether the increase in palmitic or stearic acid in the mutant lines affected the rate of the more saturated fatty acid TAG degradation.

Changes in TAG Content of the Cotyledon during Germination. Seeds with similar genetic backgrounds and sizes were selected for each twin experiment. As Figure 1 shows, mutant lines had a behavior similar to that of the corresponding control, the observed differences being more related to the line background than to the mutant phenotype. Thus, each mutant line catabolized the TAG at a rate that was similar to that of the corresponding control. The maximum TAG degradation rate in all lines was found between 0 and 2

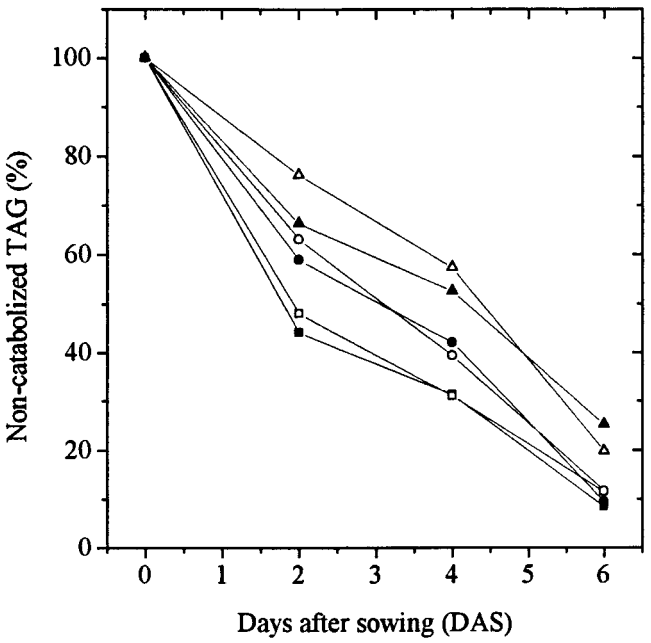

Figure 1. Noncatabolized TAG during seed germination of mutant lines CAS-3 (□), CAS-5 (●), and CAS-12 (▲) and their respective control lines RHA-274 (■), CAS-6 (○), and G-8 (△).

Table 2. Individual TAG Species Included in Each Group Used in This Study[a]

group	TAG species included
PP_	POP, PLP, PPoP,[1] PAsP[1]
SS_	SOS,[2] SLS[2]
OO_	POO, PoOO,[1] SOO, OOO, OOAs,[1] OOL, OOA,[3] OOB[3]
LL_	PLL, PoLL,[1] PlLL,[1] SLL, OLL, AsLL,[1] LLL, LLA[2]
Sat.Sat._	POS, PLS, POB,[1] PLB,[1] SLA,[2] SLB[2]
Sat._ _	PPoL,[1] PPlL,[1] POL, POAs,[1] SOPo,[1] SOL, SAsL,[1] AOL[2]

[a] Some TAG species are found only in some phenotypes: [1] high palmitic acid mutants, [2] high stearic acid mutants, and [3] high oleic acid mutants.

DAS, ranging between 25 mg TAG (mg of FW)$^{-1}$ (day)$^{-1}$ in G-8 and 55 mg TAG (mg of FW)$^{-1}$ (day)$^{-1}$ in RHA-274. At 6 DAS, most of the TAG were already degraded, the levels of TAG in mutant lines CAS-3 and CAS-5 and their corresponding controls being near 15% of the initial TAG content, whereas the levels found in mutant CAS-12 and the high-oleic control were around 25% the initial content, although in this case the control had more remaining TAG than the mutant line.

Changes in TAG Species during Sunflower Seed Germination. To study the utilization of the different TAG species during seed germination, the TAG composition has been determined by GLC at three stages of seedling development: dry seeds, 2 DAS, and 6 DAS. As previously described (Fernández-Moya et al., 2000), >36 TAG species can be determined in these lines. We have grouped those species into six groups according to their fatty acid compositions (see Table 2) to determine whether there were any divergences in the different TAG species degradation, mainly the ones with two saturated fatty acids.

Table 3 shows the evolution of these groups during the germination of control and mutant lines. At 2 DAS some important differences could be found; the TAG molecules with at least two molecules of oleic acid (OO_) are better degraded in all cases, control and mutant lines, than those with at least two molecules of linoleic

Table 3. TAG Composition in the Dry Seed (0) and at 2 and 6 DAS of Mutant Lines CAS-3, CAS-5, and CAS12 and Controls RHA-274 (a Standard Fatty Acid Composition Line) and G-8 (a High-Oleic Line)[a]

		TAG group (mol %)					
line	DAS	PP_	SS_	OO_	LL_	Sat.Sat._	Sat._ _
RHA-274	0	1.2	–[b]	27.5	58.0	1.7	11.6
	2	0.9	–	24.0	62.9	1.3	10.9
	6	0.6	–	25.7	62.5	1.0	10.2
G-8	0	–	0.5	99.0	–	0.4	–
	2	–	0.5	98.9	–	0.7	–
	6	–	0.6	98.3	–	1.1	–
CAS-3	0	0.8	13.5	4.8	55.6	8.8	16.4
	2	0.8	13.2	3.9	59.4	8.3	14.4
	6	0.9	16.3	4.7	52.3	10.8	15.0
CAS-5	0	15.8	–	–	62.3	4.1	17.8
	2	17.8	–	–	59.2	4.5	18.5
	6	18.3	–	–	57.5	5.4	18.8
CAS-12	0	12.4	–	72.2	–	3.2	12.3
	2	13.5	–	69.8	–	3.3	13.4
	6	12.4	–	70.9	–	3.6	13.1

[a] TAG groups are as in Table 2. Mean of two independent experiments; SD < 10% of the mean value. [b] <0.5%.

acid (LL_). The high-oleic control line G-8, in which OO_ is almost the only type of TAG available (99% of TAG), also shows that triolein is more rapidly degraded in this line, from 71.2 to 68,7%, than the other minority TAG forms (POO, SOO, or LOO) (data not shown). In the high-palmitic mutant CAS-5, which has no OO_, the LL_ is the class that decreased more quickly, from 62.3 to 59.2%.

TAG molecules with two palmitic acids are not well catabolized in the high-palmitic mutants CAS-5 and CAS-12. The levels of PP_ in CAS-5 changed from 15.8 to 17.8%, whereas those in CAS-12 changed from 12.4 to 13.5% during the first 2 DAS. The group with one saturated fatty acid and two unsaturated ones (Sat._ _) showed a behavior similar to that of the PP_ class in the high-palmitic lines. However, normal or high-stearic lines showed the opposite tendency. Whereas in RHA-274 and CAS-3 this group (Sat._ _) is mostly formed by POL and SOL (95%) and the best catabolized TAG accounting for all of the observed variation is the SOL species in CAS-5 and CAS-12, the SOL species is a minor (0.4% in CAS-5) or absent (in CAS-12) component and most of the TAG in this group contain palmitic acid as the saturated fatty acid (Fernádez-Moya et al., 2000).

At 6 DAS most of these differences had disappeared, but the mutant lines CAS-3 and CAS-5 showed lower degradation of some TAG species: (i) PP_ in CAS-5 increased from 15.8 to 18.3% due to the PLP species; (ii) SS_ increased from 13.5 to 16.3% in CAS-3 due to the SLS species; and (iii) Sat.Sat._ increased in both mutants, CAS-3 and CAS-5, due to SLA/SLB and PLS/PLB, respectively. In all of these cases the TAG species that were worst degraded were those bearing linoleic as unsaturated fatty acid.

DISCUSSION

Data in Figure 1 and Table 1 show that the profiles of fatty acid composition in TAG and PL as well as the evolution of the total content in TAG during seed germination are similar in all lines. The evolution of the total content in the high-oleic lines, G-8 and CAS-

12, is slightly retarded, as previously described (Rodríguez-Rosales et al., 1998) for another high-oleic hybrid sunflower.

Our results indicate that there are two different catalytic phases during the early sunflower germination stages. The initial phase is characterized by a strong TAG degradation with a preference for TAG species containing at least two oleic acid molecules as well as for the SOL species. During this phase, degradation of some TAG species takes place at a lower rate; these are the ones containing one or more palmitic acid molecules or one or more linoleic acid molecules. The second phase shows a lower TAG degradation rate and relatively no specificity with respect to the substrate. During this phase, TAG species that are more extensively degraded, depending on the line, were those containing two molecules of saturated fatty acid and one linoleic acid molecule (PP_, SS_, PLS, PLB, SLA, or SLB).

The existence of more than one lipase during seed germination showing different substrate specificity and temporal expression has been proposed previously by other authors (Muto and Beevers, 1974). Our observation of a higher lipase activity during the initial period of germination (2 DAS) is in good agreement with the data presented by Arribére et al. (1994) in studies of the lipolytic system during sunflower seed germination in control lines. Those authors, however, found an accumulation of oleic-containing TAG species and a high rate of degradation of saturated-rich TAG species. The fact that the HPLC method used for separation of TAG identified only five TAG species in normal sunflower lines can account for the difference between their work and the studies we present. Our TAG separation method identifies 14 TAG species in normal sunflower lines (Fernández-Moya et al., 2000).

The catalytic preference for specific TAGs during germination in our lines is similar to that found by Okagami and Terui (1996) studying germination on *Dioscorea tokoro* seeds at 20 °C and different from the results obtained by Yoshida (1984) working with soybean seeds or those from *Pinus edulis* seeds (Hammer and Murphy, 1994). The fact that normal sunflower RHA-274 did not show greater degradation of linoleic-containing TAG and mutant lines did not show preferences for the TAG containing the predominant fatty acid goes against the proposal of Lin and Huang (1986).

The differences in TAG degradation that we observe in the high-saturated mutant lines at 6 DAS do not affect the germination process. The higher increase in percentage at 6 DAS occurs in the SS_ group, which increased 2.8%. Taking into account that the remaining TAG in this line at 6 DAS was only 11.4% of the initial TAG found in the seed, this increase in SS_ is only 0.32% of the initial TAG. The other differences found in the degradation rate of TAGs are in a similar range and are thus not important in absolute value.

In a previous work (Fernández-Moya et al., 2000) we have found that mutant lines had increased levels of TAG species SLL (high-stearic lines) and PLL (high-palmitic line) with respect to the levels expected from pancreatic lipase analysis. These TAG species are degraded very slowly during the initial phase of germination, which we have otherwise characterized as the phase with the highest rate of TAG degradation. A "safety" mechanism could be operating in sunflower. During seed formation, abnormally high levels of particular fatty acids (i.e., saturated ones in the case of our mutant lines) are excluded from TAG species that will be preferentially degraded at the beginning of seed germination (OO_ species).

In this way a fast initial rate of germination would be warranted independent of the seed composition. Once the early steps of germination have taken place, the rest of the TAG could be degraded, although at a slower rate. The difficulties observed in germination of some oilseeds with high-saturated acid phenotype, such as high-stearic acid soybean (*Glycine max*) hybrids obtained from crosses between two high-stearic mutants (Rahman et al., 1997), could be due to the lack of such a mechanism, so that "nondesirable" TAG are accumulated during germination.

ABBREVIATIONS USED

DAS, days after sowing; TAG, triacylglycerols; PL, polar lipids; P, palmitic acid (C16:0); Po, palmitoleic acid (C16:1); Pl, palmitolinoleic acid (C16:2); S, stearic acid (C18:0); O, oleic acid (C18:1); As, asclepic acid (C18:1A); L, linoleic acid (18:2); Ln, linolenic acid (C18:3); A, araquidic acid (C20:0); B, behenic acid (C22:0).

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