Lipid Characterization in Vegetative Tissues of High Saturated Fatty Acid Sunflower Mutants

Sara Cantisán, Enrique Martínez-Force, Rosario Álvarez-Ortega, and Rafael Garcés*

Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Modifications of the fatty acid composition of plant vegetative tissues produce deficient plant growth. To determine the expression of the seed high-saturated sunflower (*Helianthus annuus* L.) mutant character during the vegetative cycle, five sunflower mutant lines (three high-stearic and two high-palmitic) have been studied during their germination and vegetative cycle. No significant variations with regard to the control lines were observed in the mutant vegetative tissue lipids; however, during seed germination important differences between lines were found. Although in the early steps of germination the palmitic and stearic acid levels in the respective mutants seedling cotyledons continued being higher than those of the control lines, they decreased and reached values similar to the controls, except in CAS-3. Variations in the cotyledon palmitic acid content with regard to the control line were also observed in high-stearic mutants, suggesting the expression of a modified acyl-ACP thioesterase or recycling of seed fatty acids during seedling development.

Keywords: *Helianthus annuus; palmitic acid; plant lipid metabolism; saturated fatty acids; stearic acid; sunflower*

INTRODUCTION

During seed germination, the sunflower embryo makes use of both lipids, mainly triacylglycerols, and reserve proteins stored in the cotyledons for its growth. The composition of seed reserve lipids does not resemble that of vegetative tissues. Good germination is, therefore, determined by the ability to catabolize these reserves and make the new fatty acids by de novo synthesis. Some plant varieties with a fatty acid composition altered by breeding, mutagenesis, or genetic engineering present growth problems during germination or in the vegetative cycle. These problems have been associated with the expression of the mutant character during these periods. For instance, an *Arabidopsis thaliana* mutant with a high stearic acid content in its seed lipids (James and Dooner, 1990) presented a miniature growth associated with the expression of the mutant character in leaves (Lightner et al., 1994a). Furthermore, seeds of a new line of canola, rich in stearic acid, have marked problems associated with the new character during germination, those with highest values being not even able to germinate (Thompson and Li, 1997). A high-oleic mutant of *Arabidopsis*, which presented poor germination and development at low temperature, has also been obtained (Miquel and Browse, 1994). Nevertheless, other genetically modified lines do not present these anomalies: high-oleic sunflower (Soldatov, 1976), linseed with a low linolenic acid content (Green, 1986), or canola with a high lauric acid content (Eccleston et al., 1996). In the case of both sunflower and linseed, mutant seed selection was always made after countryside field growth, to prevent the selection of deficient plants. Partly as a consequence of this, the new character might only be expressed by the seed (Garcés et al., 1989; Tonnet and Green, 1987), suggesting the existence of specific seed isoenzymes in at least some of the fatty acid metabolic reactions in these species. Thus, by determining the expression of this new mutant character in the vegetative tissues, good development of these new mutant lines for future agricultural use can be forecasted. Our hypothesis is that, because these new mutants have been always selected in the field, no altered morphology or development will be found. This work studied the catabolism of the seed storage lipids in the cotyledon and the de novo fatty acid synthesis in the different vegetative tissues and cotyledon during the germination of mutant sunflower seeds with a high saturated fatty acid content.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Sunflower (*Helianthus annuus* L.) seeds from the mutant lines CAS-3, CAS-4, and CAS-8, with a high stearic acid content, and CAS-5 and CAS-12, with a high palmitic acid content (Osorio et al., 1995; Fernández-Martínez et al., 1997), were used in this work. The control seeds were from normal fatty acid content line RHA-274 and from the high-oleic mutant HA-OL9 (Fernández-Martínez et al., 1993) derived from Pervenets (Soldatov, 1976). Seeds were sowed on vermiculite, and samples of cotyledon, hypocotyl, stem, root, and leaves were taken up to 30 days after sowing (DAS). Plants were cultivated in growth chambers at temperature of 25/15 °C (day/night), 16 h photoperiod, and 300 μEinstein m^{-2} s^{-1} light intensity. Samples from three different plants were analyzed for each experiment.

Lipid Extraction and Separation. Peeled seeds and vegetative tissues were ground in a glass tube with sand. Total lipids were extracted into hexane/2-propanol (Hara and Radin, 1978). Lipids were separated into triacylglycerols, diacylglycerols, and polar lipids fractions on TLC (Henderson and Tocher, 1992) silica gel 60 plates, thickness = 0.25 mm, developed with hexane/ethyl ether/formic acid (75:25:1, by vol). Lipid fractions were scraped off the plates and eluted from silica with chloroform/methanol (1:2, v/v). Polar lipids were separated on TLC silica gel 60 plates developed with chloroform/

* Author to whom correspondence should be addressed (telephone 34-954611550; fax 34-954616790; e-mail rgarces@cica.es).

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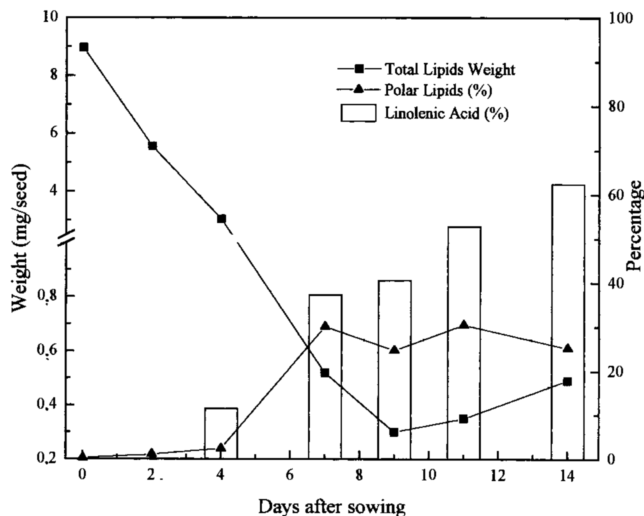

Figure 1. Changes in oil weight (■), percentage of polar lipids (▲), and percentage of linolenic acid (as white bars) in the oil of germinating seeds of the control line RHA-274. SD <10% of mean value.

methanol/acetic acid/H_2O (85:15:10:3.5, by vol) or $(NH_4)_2SO_4$-impregnated silica gel plates (Khan and Williams, 1977) developed with acetone/benzene/H_2O (90:30:10, by vol). Polar lipids were identified by comparison with known standards (purchased from Sigma).

Lipid Analysis. To quantify the total oil content, lipids were weighed after the initial extraction. For quantitative determination of isolated lipid classes, heptadecanoic acid (17:0) was added as an internal standard before GLC analysis. Fatty acid methyl esters were obtained from total or isolated lipids (triacylglicerides and, total and individual, polar lipids) by heating the samples at 80 °C during 1 h in a 3 mL solution of methanol/toluene/H_2SO_4 (88:10:2) (Garcés and Mancha, 1993). After cooling, 1 mL of heptane was added and mixed. The fatty acid methyl esters were recovered from the upper phase. Fatty acid methyl esters were separated on a Supelco SP2380 capillary column (30 m length; 0.32 mm i.d.; 0.20 mm film thickness) of fused silica (Bellefonte, PA) and quantified by hydrogen flame ionization detection (FID) using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA). Hydrogen was used as carrier gas, the linear gas rate being 28 cm/s. The injector and detector temperatures were 220 °C, oven temperature was 170 °C, and the split ratio was 1:50. Fatty acids were identified by comparison with known standards (purchased from Sigma).

RESULTS

Fatty Acid Composition during Germination in the High-Stearic Mutant Cotyledons. As lipids are hydrolyzed during seed germination, de novo fatty acid synthesis for new lipids, mainly membrane ones, takes place. In the seedling cotyledon of a normal sunflower line, such as RHA-274, the oil content decreases by ~95% during the first 9 days. Meanwhile, the polar lipid content increases to up to 30% of the total lipids at 14 DAS (Figure 1); mutants seedlings have the same behavior. During this period the cotyledon became green, part of this process being active synthesis of linolenic acid, an essential fatty acid for photosynthetic tissues (Webb and Green, 1991); this occurs to a lesser extent in mutant cotyledons (Figures 2B and 3B). In all cases the corresponding decrease is found in oleic and linoleic acids, which were the main fatty acids in seed oils. A wide variation in these decreases depending on seed type was found.

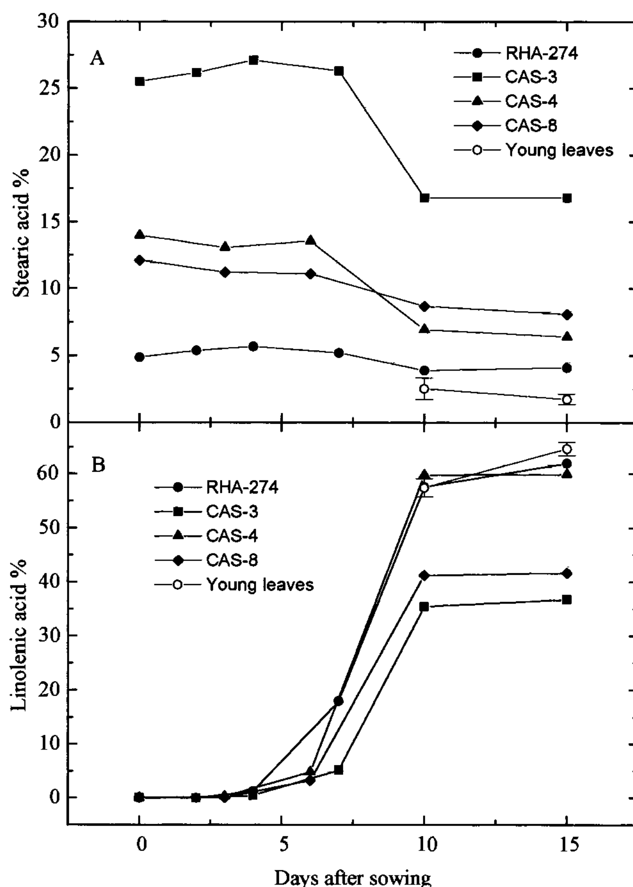

Figure 2. Variation of stearic acid content (A) and linolenic acid content (B) in the germinating cotyledons and leaves: control line, RHA-274 (●), and the high-stearic mutants CAS-3 (■), CAS-4 (▲), and CAS-8 (◆). Leaf values (○) are shown as a single value obtained as the mean of all the sunflower lines with standard deviation as error bars. Cotyledons SD <10% of mean value.

The relative amount of stearic acid in cotyledons of the high-stearic mutants remains unchanged up to 7 DAS (Figure 2A). From that moment onward and coinciding with the practical disappearance of the reserve lipids (Figure 1), it decreases to 6.4% in CAS-4 and to 8.1% in CAS-8, near the value found in the control (4%). CAS-3 presents a more marked decrease (9%) but maintains a stearic acid level 4 times higher than that normally observed in the control line. No changes were found from 10 DAS in the amount of stearic acid. The same figure represents the content of stearic acid of the first leaves at 10 and 15 DAS. Given that both the mutant and the control leaves presented very similar values of stearic acid, the mean values of all lines and the standard deviations as error bars are shown.

At the same time, from 7 DAS, the linolenic acid content increases in the cotyledons (Figure 2B). In this case, CAS-3 and CAS-8 have a lower content than the control line RHA-274. The same figure displays the mean linolenic acid content of young leaves, assessed from the data of all lines. As above, no significant differences were found between the normal leaves and any of the mutant leaves.

One of the most important differences between high-stearic mutants and control lines (Table 1) is their smaller content of palmitic acid in cotyledons at 15 DAS. Whereas in the control line this fatty acid increases during the germination period, from 6.4% in the dry

Table 1. Fatty Acid Composition of Seeds (0 DAS) and Seedling Cotyledons (15 DAS) from Control (RHA-274 and HA-OL9) and Mutant Lines[a]

		fatty acid composition (mol %)							
DAS	line	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
0	RHA-274	6.4		4.9	30.8	56.2		0.3	1.0
	CAS-3	5.5		25.5	17.1	48.1		1.4	1.5
	CAS-4	5.4		14.0	31.4	47.2		0.7	1.5
	CAS-8	5.4		12.1	28.2	52.9		0.3	0.8
	CAS-5	30.7	4.4	4.3	8.1	50.8		0.2	1.2
	HA-OL9	5.2		3.7	88.1	1.5		0.3	1.1
	CAS-12	27.8	7.6	1.7	56.7	4.4		0.3	1.3
15	RHA-274	12.1		4.1	1.0	17.1	62.0		3.0
	CAS-3	8.7		16.8	4.8	29.8	36.8	1.1	1.8
	CAS-4	6.1	0.8	6.4	2.0	22.7	60.0	1.4	0.7
	CAS-8	6.3	1.0	8.1	10.0	30.1	41.7	0.9	1.9
	CAS-5	17.8	2.1	4.0	6.0	24.6	43.4	1.0	1.1
	HA-OL9	14.0	2.2	1.6	1.3	17.2	61.9	0.2	1.6
	CAS-12	18.0	4.3	1.8	5.7	24.5	40.6		5.0

[a] 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:0 araquidic acid; 22:0, behenic acid. DAS, days after sowing. Data represent the mean of three replicates. SD <10% of mean value.

seed to 12.1% in 15 DAS RHA-274 cotyledon, in the high-stearic mutants it remains almost constant, from ~5% in the dry seeds to only 6–8% in the 15 DAS cotyledons.

Fatty Acid Composition during Germination in the High-Palmitic Mutant Cotyledons. The same experiment was carried out with the high-palmitic mutants, CAS-5 and CAS-12, and the high-oleic mutant line, HA-OL9, which serves as control for CAS-12. The vegetative tissues in the control lines present a high palmitic acid content (~15%); thus, beyond 7 DAS the level of this fatty acid in the cotyledon decreases in the mutants and increases in controls until reaching a medium level (Figure 3A). As above, the mean value with the standard deviation bars of palmitic acid content of young leaves beyond 10 DAS is shown in Figure 3A. As in the case for high stearic acid lines, the palmitic acid content begins to decrease as the content of linolenic acid increases beyond 7 DAS (Figure 3B).

The delay in CAS-5 is similar to that in the high stearic acid mutants. In the case of the high-oleic Soldatov mutant and our high-palmitic and high-oleic line, CAS-12, the delay is higher; in any case, the three mutant lines arrived at a similar relative amount of linolenic acid. In this case significant differences in the linolenic acid content of young leaves were found. For this reason the mean linolenic acid contents of CAS-5 and CAS-12 were represented independently of those of RHA-274 and HA-OL9. The very young leaves of the high-palmitic mutants present less linolenic acid than the control lines, although beyond 20 DAS all of them had normal values.

Fatty Acid Composition in Leaves. Some mutants—such as the high-stearic mutant of *Arabidopsis*—present high values of this fatty acid in leaf lipids (Lightner et al., 1994b). Thus, the compositions of fatty acids of the main polar lipids of the leaves (7–8 weeks after sowing) have been studied. Table 2 shows the fatty acid composition of the principal leaf polar lipids: digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), and phosphatidylglycerol (PG); these lipids account for 75–80% of total leaf lipids [see review by Harwood (1980)]. The content of 18:3 in MGDG, usually rich in this fatty acid, is between 90.5% in CAS-12 and 94.3% in CAS-3, the control line having a value of 92.3%.

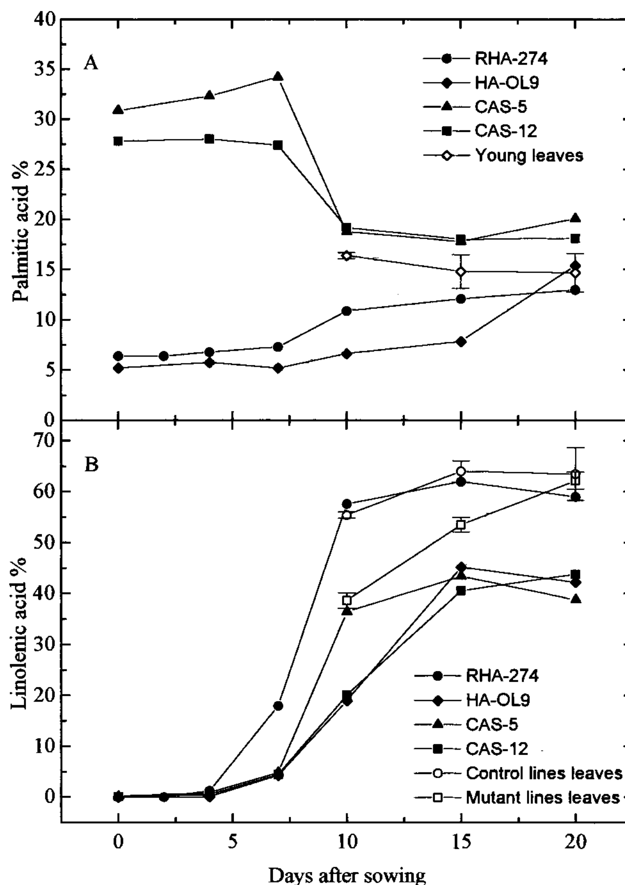

Figure 3. Variation of palmitic acid content (A) and linolenic acid content (B) in germinating cotyledons and leaves: control lines, RHA-274 (●) and HA-OL9 (◆), and the high-palmitic mutants CAS-5 (▲) and CAS-12 (■). Leaf palmitic values (◇) are shown as a single value obtained as the mean of all the sunflower lines with standard deviation as error bars. Leaf linolenic values from control (○) or mutant lines (□) are shown as leaf palmitic values. Cotyledons SD <10% of mean value.

The palmitic acid and stearic acid contents in all cases are very low irrespective of the genotype. DGDG is also rich in 18:3, although in this case there is more variability, however, unrelated to the mutations. The highest value of stearic acid is found in the high-oleic line HA-OL9, and that of palmitic acid appeared in CAS-4 (high stearic). The last lipid of leaves presented is PG, which is characterized by a high content in 16:1 Δ3-trans (Webb and Green, 1991). In our case, the levels of this fatty acid ranged between 27.8% in CAS-12 and 37.1% in RHA-274. The highest values of stearic and palmitic acids are, in both cases, in the high-oleic line HA-OL9.

Fatty Acid Composition in Other Vegetative Tissues. In this research, the fatty acid compositions of both the root and the stem were studied during germination, in mutants, and in controls. Results are displayed in Table 3. Concerning the high stearic acid mutants (Table 3A), CAS-3 and CAS-8 presented a small increase of stearic acid content at 10 DAS in their roots; nevertheless, at 30 DAS, CAS-3 presented a level similar to that of the control line, a little higher in the case of CAS-8. In the high-palmitic mutants (Table 3B) there are no variations in the content of this fatty acid at 10 and 30 DAS. The data obtained for hypocotyl and stem do not show any correlation with the studied mutations (Table 3).

Table 2. Fatty Acid Composition of Leaf Main Polar Lipids from Control (RHA-274 and HA-OL9) and Mutant Lines[a]

line	lipid	fatty acid composition (mol %)					
		16:0	16:1	18:0	18:1	18:2	18:3
RHA-274	MGDG	1.9	0.3	0.3	0.4	4.8	92.3
	DGDG	9.6	1.6	1.1	0.7	4.6	82.8
	PG	14.2	37.1	2.8	2.3	14.9	28.6
CAS-3	MGDG	1.2	0.1	0.3	0.1	4.0	94.3
	DGDG	6.6	2.5	2.7	0.4	4.9	82.9
	PG	14.5	29.3	4.2	2.2	10.5	39.3
CAS-4	MGDG	1.7	0.2	1.1	0.6	4.0	92.5
	DGDG	16.6	0.5	2.5	1.6	10.1	68.7
	PG	12.6	31.3	2.9	3.6	11.1	38.7
CAS-8	MGDG	2.2	0.3	0.5	0.2	4.1	92.8
	DGDG	14.6	0.1	2.4	0.6	9.8	72.5
	PG	12.4	32.4	2.5	2.9	10.4	39.5
CAS-5	MGDG	1.3	0.2	0.2	0.5	5.2	92.6
	DGDG	10.3	2.1	2.3	1.5	3.9	79.9
	PG	12.5	30.5	1.5	3.3	15.4	36.8
HA-OL9	MGDG	1.3	0.1	0.3	1.3	5.1	91.9
	DGDG	11.0	0.3	5.4	0.9	3.4	79.1
	PG	19.1	30.1	4.3	8.4	10.9	27.2
CAS-12	MGDG	1.3	0.2	0.2	0.6	7.2	90.5
	DGDG	9.2	0.4	2.3	1.8	6.1	80.4
	PG	15.2	27.8	2.0	4.1	18.3	32.7

[a] 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol. Data represent the mean of three replicates. SD <10% of mean value.

Table 3. (A) Stearic Acid Content of Vegetative Tissues of Control (RHA-274) and High-Stearic Mutants and (B) Palmitic Acid Content of Vegetative Tissues of Control (RHA-274 and HA-OL9) and High-Palmitic Mutants[a]

line	root		hypocotyl	stem
	10 DAS	30 DAS	10 DAS	30 DAS
(A) Stearic Acid Content (mol %)				
RHA-274	2.7	2.2	3.4	2.6
CAS-3	5.1	2.7	6.9	3.8
CAS-4	1.9	1.8	3.1	2.6
CAS-8	6.8	5.0	6.2	4.0
(B) Palmitic Acid Content (mol %)				
RHA-274	26.6	25.5	19.5	22.6
HA-OL9	23.7	23.8	16.7	25.7
CAS-5	24.6	24.4	17.6	23.0
CAS-12	26.1	28.8	21.3	26.0

[a] DAS, days after sowing. Data represent the mean of three replicates. SD <10% of mean value.

DISCUSSION

The fatty acid sunflower mutants, such as the high-oleic line (Soldatov, 1976) and those studied in this work, were selected after chemical or physical mutagenesis on seeds, by field growth, and selecting only those plants with a good development. Therefore, they do not present problems during germination or at any other period of the vegetative cycle; the opposite happens in other mutant lines in which the expression of the mutant character during the vegetative cycle produces disorders in the normal metabolism (James and Dooner, 1990; Miquel and Browse, 1994; Thompson and Li, 1997). Genetic studies of these mutants show that two genes are needed to control the high stearic acid character (Fernandez-Martínez et al., 1998) and three are needed in the case of the high-palmitic mutants (Perez-Vich et al., 1998).

Each vegetative tissue has a characteristic fatty acid composition, the photosynthetic ones having a very high content of linolenic acid, whereas the others, such as the root, show a higher content of linoleic acid [see review by Harwood (1980)]. All of these tissues also have a high content of palmitic acid. The data obtained after the analysis of lipids from mutant vegetative tissues suggest that the mutant character is not expressed in them, except in the case of the cotyledon during germination. In this case the analysis of lipids suggests that at least one of the altered genes involved in the mutant phenotype could be expressed during germination in the high-stearic mutants.

The increase of palmitic acid found in the 15 DAS seedling cotyledons of RHA-274 with regard to the seed could be due to two enzymatic activities: (i) an isoenzyme of the β-ketoacyl-ACP synthetase II (KAS II), different from the one found in seeds, having a lower affinity or activity on the palmitoyl-ACP; or (ii) an acyl-ACP thioesterase (probably of the FatB type) with a higher activity on palmitoyl-ACP (Jones et al., 1995). However, in the cotyledon of the high- or medium-stearic mutants there is a lower content of palmitic acid (Table 1) at the same time that the stearic acid content is higher than normal (Figure 2A). Because in these mutant lines there is not a complete change of phenotype from mutant to normal during development of seedling cotyledons, the expression of one of the altered genes in the high-stearic mutants could still be producing an interference on the normal fatty acid composition of the cotyledons. Although the expression of a modified stearoyl-ACP desaturase with lower activity in the cotyledon would likely be phenotypically recessive, with any of the possible mechanisms involved in the higher palmitic acid content found in control cotyledons, a modified acyl-ACP thioesterase with lower activity over palmitoyl-ACP or/and higher on stearoyl-ACP should be dominant (if this thioesterase hydrolyzes both substrates) or codominant (if more than one isoenzyme is responsible for the hydrolytic activity).

The data obtained with the high-palmitic mutant do not exclude the possibility that a thioesterase (with a high activity on palmitoyl-ACP) could be expressed during cotyledon development, because the expected phenotype (lower palmitic acid content in the seedling cotyledon than in the mutant seed but higher than in the control seedling cotyledon) is already found (Table 1). Nevertheless, the expression of a modified KAS II during the development of seedling cotyledons can be excluded, because it would produce a higher content of palmitic acid than the one observed.

Therefore, our data could be explained if there is a modified acyl-ACP thioesterase with a higher activity/affinity on saturated acyl-ACPs (FatB type) in high-stearic mutants, not only during the seed formation but also in the cotyledon during seed germination; also, it is possible that after lipase triacylglycerol hydrolysis, part of the seed 18:0 was reused for seedling polar lipid synthesis, increasing the amount of 18:0 found in comparison with the control line.

ABBREVIATIONS USED

DAS, days after sowing, DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol.

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