Fatty Acid Composition in Developing High Saturated Sunflower (*Helianthus annuus*) Seeds: Maturation Changes and Temperature Effect

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Developing seeds from sunflower high palmitic acid mutants (CAS-5 and CAS-12) showed high levels of palmitic acid at early stages. The stearic acid content of high stearic mutants (CAS-3, CAS-4, and CAS-8) increased from low or medium initial values until it reached the maximum level at 16 days after flowering. All mutant lines increased palmitic acid content at high growth temperature with CAS-5 having the maximum increase (5.7%). At the same time, all but CAS-3 increased stearic acid content at low temperature with CAS-8 showing the maximum increase (9.6%). The unsaturation level in the mutants had a different linoleic/oleic ratio than the control line, but always with a ratio higher than 1 at low temperature. Only mutant CAS-5 maintained a similar desaturation level at any temperature, being 3.5−11 times higher than that of the other lines. The temperature affects the polar lipids in a similar way.

Keywords: *Sunflower; oilseed; mutant; temperature*

INTRODUCTION

To maximize the amount of stored energy in a small tissue volume, one of the strategies more widely used by many plant species is to store oils instead of carbohydrates, because the energy produced by oxidation of fatty acids is two times higher than that from carbohydrates (Slack and Browse, 1984). Sunflower (*Helianthus annuus* L.) seed oil is mostly composed by triacylglycerides (96%) and phospholipids (1−2%); the major polar lipids being phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine (Dorrell, 1978). The oil content in sunflower seeds shows a quick increase from the 14th day after flowering (DAF) to the 35th DAF (when the seed becomes physiologically mature); from this age the oil content remains constant without any significant change (Robertson et al., 1978).

The fatty acid composition of oils, which is not constant during seed formation, is determined by a number of factors, but the genotype of the plant being the main one. In sunflower (Robertson et al., 1978) and safflower seeds (Ichiara and Noda, 1980), the linoleic acid content increases from the 14th DAF, whereas the oleic acid content decreases. At the same time saturated fatty acids show a small decrease. On the other hand, during soybean seed development, oleic and linoleic acid levels increase, palmitic and stearic levels remain constant, and myristic acid content drastically decreases (Cherry et al., 1984). Environmental factors, such as temperature and light, also affect the oil fatty acid composition. In rapeseed (Trémolières et al., 1978), the high temperature stimulates the biosynthesis of oleic acid and decreases the desaturation rate, producing an increase in oleic acid-containing triacylglycerides. In flax (Green, 1986), the high temperature produces a reduction in the unsaturation level and a decrease in linoleic and linolenic acid levels. Together with this decrement, an increase in oleic acid levels and, to a lesser extent, in saturated fatty acids can be observed. This temperature effect is also observed in normal and high oleic sunflower, where a temperature decrease produced a higher linoleic acid content and a lower oleic acid content (Harris et al., 1978; Fernández-Martínez et al., 1986). Phospholipids are directly involved in temperature response, and their fatty acid composition is the first to be modified in the membrane as a response to changes in temperature (Garcés et al., 1992). In leaves, the level of fatty acid unsaturation of phosphatidylglycerol shows a close relationship with the degree of plant chilling sensitivity. Genetically engineered plants with modified phosphatidylglycerol fatty acid composition that show a lower chilling sensitivity have been obtained (Murata et al., 1992). Additionally, it has been shown that the genes encoding some membrane-bound desaturases that act on phospholipids, like chloroplast ω3-desaturase from *Arabidopsis thaliana*, are regulated by temperature (Gibson et al., 1994). Also, ω9- and ω6-desaturases from leaves are induced in plants transferred to lower growth temperature (Williams et al., 1996).

Our group has already shown that the high saturated phenotype is observed only in seeds, but not in vegetative tissues (Cantisán et al., unpublished). In this work, the fatty acid composition in developing high saturated sunflower seeds has been analyzed to determine the timing of expression of the high saturated phenotype during seed maturation. Additionally, we report the effects of temperature on the fatty acid composition during seed formation of wild-type and mutant lines.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Sunflower (*Helianthus annuus* L.) seeds with high stearic acid content from mutant lines CAS-3, CAS-4, and CAS-8 or with high

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palmitic acid content from mutant lines CAS-5 and CAS-12 (Osorio et al., 1995; Fernández-Martínez et al., 1997) were used in this work. The control seeds were from public lines RHA-274, with normal fatty acid content, and HA-OL9 (Fernández-Martínez et al., 1993) derived from Pervenets (Soldatov, 1976) with high oleic acid content. Plants were cultivated, except when indicated, in growth chambers at 25/15 °C (day/night), 16 h photoperiod, and 300 μEinstein m^{-2} s^{-1} light intensity. Seeds for analysis were harvested from 8 to 36 DAF and processed immediately. Younger seeds were not harvested due to their nonsolid endosperms. Seeds from CAS-5, CAS-12, and HA-OL9 were collected from 12 to 36 DAF, as the first and last data points, respectively, due to their longer life cycle respect to the other lines. To determine the effect of the temperature on the fatty acid composition, plants were grown in growth chambers either at 20/10 or at 30/20 °C (day/night) with the same intensity and photoperiod as above. Seeds at 10, 20, and 35 DAF were harvested, and their lipids were analyzed. Three replicates were made for each experiment; a different capitulum was taken and analyzed independently for each replicate.

Lipid Extraction and Separation. Seeds were peeled, and about 500 mg was ground in a glass tube with sand. Total lipids were extracted following the method of Hara and Radin (1978). Lipids were fractionated into triacylglycerols, diacylglycerols, and polar lipids on TLC (Henderson and Tocher, 1992) silica gel plates (thickness 0.25 mm) developed with hexane:ethyl ether:formic acid (75:25:1; by volume). Lipid fractions were scraped off the plates and eluted from silica with chloroform:methanol (1:2; by volume). To determine their fatty acid composition, polar lipids were further separated on TLC silica gel plates developed with chloroform:methanol:acetic acid:H_2O (85:15:10:3.5; by volume) or $(NH_4)_2SO_4$-impregnated silica gel plates developed with acetone:benzene:H_2O (90:30:10; by volume) (Khan and Williams, 1977). Phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine were identified by comparison with known standards and for fatty acid composition analysis were scraped off the plates.

Lipid Analysis. Fatty acid methyl esters were obtained from lipids by heating the samples at 80 °C for 1 h in a 3 mL solution of methanol:toluene:H_2SO_4 (88:10:2; by volume) (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 (Bellafonte, 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 with a linear gas rate of 28 cm/s. The injector and detector temperature was 220 °C, with a oven temperature of 170 °C and a split ratio of 1/50. Fatty acids were identified by comparison with known standards.

RESULTS AND DISCUSSION

Saturated Fatty Acid Content during Seed Development. The saturated fatty acid content of seeds from different maturation stages was determined. The levels of stearic acid found in control line and mutants CAS-3, CAS-4, and CAS-8 during seed development at 25/15 °C (day/night) are shown in Figure 1A. While in control line, RHA-274, the stearic acid level presented a small fluctuation with a final content close to 5%, a behavior similar to that described previously by Robertson et al. (1978). In the high stearic mutant CAS-3, the stearic acid level was already about 22% at 8 DAF and then increased to 27%. Mutants CAS-4 and CAS-8 showed a normal stearic acid level at 8 DAF (3 and 6%, respectively), and then the level increased to 15%. After 16 DAF, the stearic acid levels remained almost constant in all mutants.

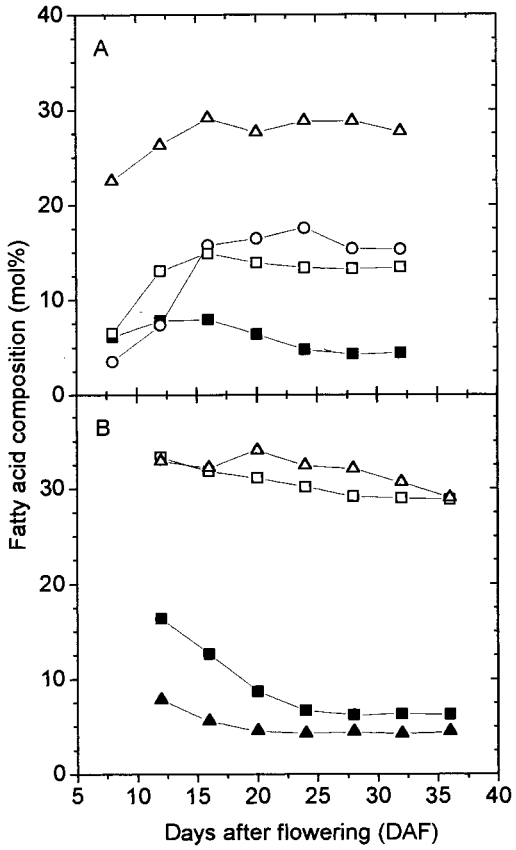

Figure 1. (A) Stearic acid content in total lipids during seed maturation in control line, RHA-274 (■), and high stearic mutants CAS-3 (△), CAS-4 (○), and CAS-8 (□). (B) Palmitic acid content in total lipids during seed maturation in control lines, RHA-274 (■) and HA-OL-9 (▲), and high palmitic mutants CAS-5 (□) and CAS-12 (△). Each value is the mean of three independent samples. SD < 2.5% of mean value.

The stearic acid accumulation temporal pattern in the high/medium stearic acid mutants is similar to the one of storage protein genes for helianthinin (the major globulin storage protein of sunflower seeds) that start at 7 DAF with a major accumulation rate from 9 to 19 DAF (Allen et al., 1985). It is possible that both systems (lipids and proteins accumulation during seed maturation) are under the same seed-specific regulatory system. In addition, the higher starting level observed in CAS-3 suggests that the mutation is on a seed constitutive gene while those responsible for CAS-4 and CAS-8 phenotype must be temporarily expressed in seed.

Figure 1B shows the palmitic acid content during seed maturation. The high palmitic mutant lines already presented a high palmitic level at 12 DAF (the first experimental data in those lines due to their longer life cycle) showing a small decrease during seed development (Figure 1B). In control lines, RHA-274 and HA-OL9, the palmitic acid content decreased from the first experimental point down to around 5%. The high palmitic acid level just from the beginning in the mutants suggest that the affected gene is constitutively expressed in seeds maintaining the palmitic acid level present in the original material (pollen grains or flowers having about 20−25% palmitic acid) (Garcés et al., 1989). The expression of all mutant characters occurs during seed formation, suggesting that they could be a good model system to study tissue-specific gene expression.

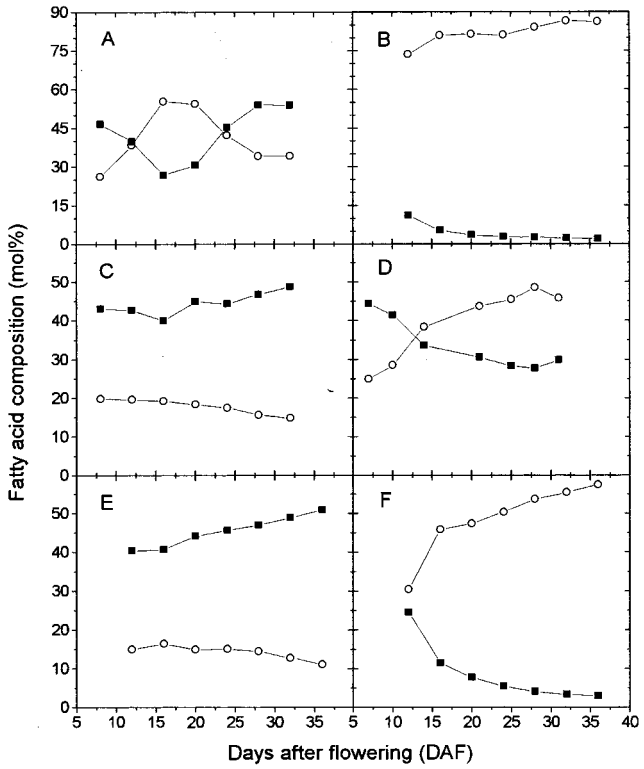

Figure 2. Evolution of total lipids unsaturated fatty acids composition during seed development in control lines, RHA-274 (A) and HA-OL9 (B), and mutant lines, CAS-3 (C), CAS-4 (D), CAS-5 (E), and CAS-12 (F) growing at 25/15 °C. Oleic acid (○); linoleic acid (■). Each value is the mean of three independent samples. SD < 2.5% of mean value.

Unsaturated Fatty Acids during Seed Development. Figure 2 shows the unsaturated fatty acid levels (oleic and linoleic acids) in mutant and control lines during seed development at 25/15 °C (day/night). The two control lines (RHA-274 and HA-OL9) showed different behaviors (see Figure 2, panels A and B). In RHA-274 (Figure 2A), oleic acid content at 8 DAF was about 26%, increased to 55.4% at 16 DAF, and then gradually decreased to 34.3% at 28 DAF. On the other hand, linoleic acid content was 46.5% at 8 DAF, decreased to 26.7% at 16 DAF, and progressively increased to 54.1% at 28 DAF. This alternated pattern between oleic and linoleic acids contents in control line is due to a delay in the induction of oleate desaturase with respect to the synthesis of the main storage fatty acid, producing an initial accumulation of oleic acid. After oleate desaturase is induced, most of the oleic acid is desaturated to linoleic acid (Garcés and Mancha, 1991). In the high oleic control line HA-OL9 (Figure 2B), oleic acid level was quite high during seed development, varying between 73% at 12 DAF and 86.3% at 36 DAF. Linoleic acid level was very low, around 11% at 12 DAF and 2% at 36 DAF. This behavior was due to the very low oleate desaturase activity of the high oleic mutant (Garcés and Mancha, 1991).

Mutant lines CAS-3 and CAS-5 (high stearic and high palmitic, respectively) contained more linoleic acid than oleic acid throughout seed development. In CAS-3 (Figure 2C), oleic acid level varied between 19.8% at 8 DAF and 14.8% at 32 DAF, while linoleic acid level varied between 43.1% at 8 DAF and 48.9 at 32 DAF. A similar behavior was observed in mutant CAS-8 but with higher levels of oleic acid (around 25% throughout seed development) due to its lower stearic acid level

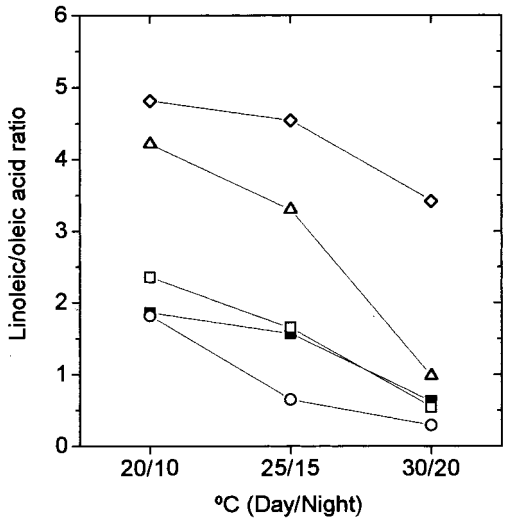

Figure 3. Linoleic/oleic acid ratio in total lipids at 35 DAF in control line, RHA-274 (■), and high saturated mutants CAS-3 (△), CAS-4 (○), CAS-8 (□), and CAS-5 (◊) growing at 20/10, 25/15, and 30/20 °C. Each value is the mean of three independent samples. SD < 2.5% of mean value.

(data not shown). In the case of mutant CAS-5 (Figure 2E), its oleic acid content varied between 14.9% at 12 DAF and 11.2% at 36 DAF, and the linoleic acid content varied between 40.4% at 8 DAF and 50.9% at 36 DAF. In contrast to CAS-3 and CAS-5, the medium stearic mutant CAS-4 showed linoleic and oleic acid levels similar to those of the control line RHA-274 at early stages of seed development (Figure 2D). Linoleic and oleic acid levels at 8 DAF were 44.5% and 24.9%, respectively. After that stage, the oleic acid content gradually increased and the linoleic acid content decreased. From 14 DAF to seed maturity, the oleic acid level was higher than the linoleic acid level, 45.9% vs 29.7% at 32 DAF seeds. Toward the end of seed maturation, the content of both fatty acids tend to cross. As mentioned before, this is also observed in control line RHA-274 (see Figure 2A) but at an earlier stage of development, suggesting a deficient induction of the oleate desaturase in mutant CAS-4. Mutant CAS-12 (Figure 2F) showed a behavior similar to the control line, HA-OL9, with a higher oleic acid content than linoleic acid content throughout seed development. The oleic and linoleic fatty acids content change between 30.4% at 12 DAF and 57.5% at 32 DAF for oleic acid and between 24.5% at 12 DAF and 2.9% at 32 DAF in the case of linoleic acid. The lower oleic acid level in CAS-12 with respect to its control line HA-OL9 was due to the higher palmitic acid content in double mutant CAS-12. The linoleic acid content, however, is similar to that of HA-OL9. Except for CAS-4 and CAS-12 (high palmitic in high oleic background), the mutants showed a higher linoleic acid percentage than oleic acid during the whole process of seed development, suggesting that in these lines the higher content in saturated lipids and, therefore, smaller membrane fluidity probably produced the induction of oleic acid desaturation (Garcés et al., 1992); hence, they do not show the transitory oleic acid accumulation. The differences between CAS-4 and the other high stearic mutants suggest the presence of different regulation patterns in the three mutants.

Influence of Temperature on Fatty Acid Composition. Growth temperature affects sunflower seed fatty acid composition (Harris et al., 1978), mainly by altering the linoleic/oleic acid ratio (unsaturation ratio).

Table 1. Total Fatty Acid Composition Observed in 35 DAF Sunflower Seeds Cultivated at 20/10 °C (Day/Night)[a]

	fatty acid composition at 20/10 °C (mol %)					
line	16:0	16:1	18:0	18:1	18:2	Rem (%)
RHA-274	5.5(−0.5)	0	5.4(−2.2)	33.7(22.2)	54.1(−19.5)	22.2
CAS-3	5.7(0.8)	0	24.5(1.1)	13.0(20.8)	54.5(−22.6)	22.6
CAS-4	4.7(1.5)	0	17.5(−5.7)	26.8(35.7)	48.6(−31.8)	37.2
CAS-8	5.6(1.4)	0	20.0(−9.6)	22.3(31.1)	52.3(−23.3)	32.5
CAS-5	25.4(5.7)	2.7(2.5)	5.5(−4.0)	11.1(2.6)	53.1(−6.8)	10.8

[a] Changes between the 20/10 and the 30/20 °C fatty acid compositions are shown in parentheses, and the total remodeled fatty acids between both temperatures, rem (%), as the sum of positive increments. Each value is the mean of three independent samples. SD < 2.5% of mean value.

Table 2. Fatty Acid Composition of Polar Lipids from 20 DAF Seeds Grown at 30/20 or 20/10 °C[a]

		fatty acid composition (mol %)									
		20/10 °C					30/20 °C				
polar lipid	line	16:0	16:1	18:0	18:1	18:2	16:0	16:1	18:0	18:1	18:2
phosphatidylinositol	RHA-274	26.1	0	14.3	13.3	46.3	21.1	0	10.3	44.5	24
	CAS-3	17.9	0	31.3	16.4	33.8	15	0	32.9	21.4	30.3
	CAS-4	19.8	0	24.7	18.3	37.2	18.2	0	21.8	43.1	16.8
	CAS-8	20.7	0	23.4	17.9	38	19.3	0	19.5	45.5	15.7
	CAS-5	36.8	0.1	4.3	5.8	51.3	38.9	0.6	2.6	7.4	50.5
phosphatidylcholine	RHA-274	12.7	0	8.3	36.9	41.3	5.2	0	3.5	73.8	17.3
	CAS-3	8.6	0	27.1	23.5	39.9	6.3	0	20	34.6	38.4
	CAS-4	9.9	0	18.1	36.3	35.7	5.4	0	12.5	71.5	10.6
	CAS-8	10.4	0	16	34.8	38.9	7.4	0	10.7	75.4	6.5
	CAS-5	27.4	3	2.3	19.3	47.2	29.6	3.4	1.5	21.6	43.9
phosphatidylethanolamine	RHA-274	14.2	0	3.5	19.2	62	14.1	0	2.4	47.8	35.3
	CAS-3	6.7	0	18.8	20	53.2	13.1	0	12.2	24.5	49.6
	CAS-4	10.5	0	10.8	24.5	54.2	13.4	0	7.2	46.4	33
	CAS-8	11	0	9.9	23.8	55.3	16.3	0	5.7	48.9	29
	CAS-5	33.2	2	0.8	7.2	55.7	30.8	2.2	1.3	9.9	55.5

[a] Each value is the mean of three independent samples. SD < 2.5% of mean value.

For instance, linoleic acid content in cultivated sunflower seeds changes from 48.7% in southern Spain (warmer weather) to 70.2% in the north (colder weather), showing a direct relationship with the average temperatures of the area and the local climatic conditions during seed development (Lajara et al., 1990). The linoleic/oleic acid ratio at 35 DAF is shown in Figure 3 for control line RHA-274 and mutant lines CAS-3, CAS-4, CAS-8, and CAS-5 cultivated at different temperatures. The control line showed a decrease in this ratio at 30/20 °C as a consequence of the decrease in the oleate desaturation, the ratio value being below 1 (three times lower than at low temperature). All the mutants except the high palmitic mutant CAS-5 and the medium stearic CAS-4 had a behavior similar to that of the control line, unsaturation ratios markedly decreasing as the temperature increases. In the case of CAS-5, this ratio diminished slightly when growth temperature increases, always having more linoleic acid than oleic acid; at high temperature there is 3.4 times more linoleic acid than oleic acid. Mutant CAS-4 had the opposite behavior, being the linoleic/oleic acid ratio below 1 from 25/15 °C.

Growth temperature also affects, although to a lesser extent, the stearic acid levels in sunflower seeds. Stearic acid contents progressively decrease (from 5.6 to 3.2%) with increasing growth temperatures (Lajara et al., 1990). In soybean, however, the opposite phenomenon has been observed (Rennie and Tanner, 1989), that is, the higher the growth temperature, the higher the stearic acid contents. Table 1 shows fatty acid composition in sunflower seeds cultivated at different temperatures. As expected, in control line RHA-274, the stearic acid content decreases from 5.4 to 3.2% when growth temperature increases. The palmitic acid level remains constant around 5% at both temperatures. All mutants lines show a slight change in palmitic acid at high growth temperature, reaching 5.7% in the case of the high palmitic mutant CAS-5. All mutant lines, except CAS-3, exhibit a higher stearic acid content at low temperature, decreasing by 9.6% in some cases (CAS-8). Mutant CAS-3, however, shows a very similar stearic acid content in both cases (24.5% at low temperature and 25.6% at high temperature). Fatty acid composition is very stable in CAS-5 (remodeling only 10.8%) in contrast to CAS-4 (remodeling 37.2%) (Table 1).

The changes in fatty acid composition observed when growth temperatures are higher have their origin in the changes suffered by several enzymatic activities. Our data suggest a decrease in oleic desaturase and a slight decrease in KAS II in all lines. On the other hand, stearic desaturase activity should increase in all mutant lines, except for CAS-3. Since this last enzymatic activity is also responsible for the palmitic desaturation (Cheesbrough and Cho, 1990), an increase in palmitoleic acid should be observed in mutant CAS-5. Table 1 shows that this is the case. The observation that the stearic desaturase activity does not increase in the case of CAS-3 points to this enzyme as responsible for the mutant phenotype of this line. In the case of CAS-5, its response to changes in temperature seems to be linked to its high palmitic phenotype. In this case, the decrease in linoleic acid content when temperature increases is somewhat slowed by its higher palmitic acid content. The fact that this phenomenon is not observed in the high stearic mutant line (CAS-3), with a saturated fatty acid content that is similar to that of CAS-5, suggests that the mechanisms that sense the degree of saturation in membranes respond more specifically

to the palmitic acid content than to that of stearic acid. Also, the general increase in palmitic acid observed in all mutant lines is magnified in this mutant, since it exhibits a low KAS II activity (Álvarez-Ortega et al., unpublished). Additionally, the increase in palmitic acid due to the temperature increase observed in CAS-5 leads to an increase in its desaturation, competing with stearic acid and thus provoking a higher palmitoleic accumulation.

The mature seeds from mutant lines with higher levels of saturated fatty acids, CAS-3 and CAS-5, would have stable levels of these acids despite the changes in growth temperature. However, seeds from mutant lines CAS-4 and CAS-8 would exhibit higher stearic acid levels at low growth temperature. From an applied point of view, low growth temperature leads to higher saturated fatty acid contents.

Effect of the Temperature on Main Polar Lipids. The temperature effect on the composition of polar lipids has been studied at 20 DAF, a period of the seed formation with an active lipid biosynthesis, when the polar lipids are metabolically active. Table 2 shows the fatty acid composition of the major polar lipids found in seeds: phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine.

Changes in these polar lipids are very similar to those found in total lipids: (i) great stability in the CAS-5 fatty acid composition; (ii) decrease in the linoleic acid content and increase in the oleic acid content with increased growth temperature (a smaller increase in CAS-3 and CAS-5); (iii) increase in the stearic acid level with lower temperature, except in phosphatidylinositol from CAS-3 and for phosphatidylethanolamine from CAS-5 (different from the results observed in total lipids); and (iv) decrease in the palmitic acid content of phosphatidylinositol and phosphatidylcholine in all mutant lines except for CAS-5, which shows an increase with the temperature increase.

In general, all the increases or decreases observed for a particular fatty acid are similar in all the mutant lines except in the case of the mutant with specific high content for that fatty acid. Further studies of the responses to growth temperature should allow us to determine the role of each polar lipid in adaptation to different temperatures. The mutants described should be a good model for the study of the temperature effects on the fatty acid composition of sunflower seeds as well as for determination of the sensor involved in adaptation to different temperatures.

ABBREVIATIONS USED

DAF, days after flowering; KAS II, β-ketoacyl synthetase II.

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