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Characterization of Sphingolipids from Sunflower Seeds with Altered Fatty Acid Composition

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ABSTRACT: Sphingolipids are a group of lipids that are derived from long-chain 1,3-dihydroxy-2-amino bases and that are involved in important processes in plants. Long-chain bases are usually found bound to long-chain fatty acids forming ceramides, the lipophilic moiety of the most common sphingolipid classes found in plant tissues: glucosyl-ceramides and glucosyl inositol phosphoryl-ceramides (GIPCs). The developing sunflower seed kernel is a tissue rich in sphingolipids, although, importantly, its glycerolipid composition can vary if some steps of the fatty acid synthesis are altered. Here, the sphingolipid composition of the seed from different sunflower mutants with altered fatty acid compositions was studied. The long-chain base composition and content were analyzed, and it was found to be similar in all of the mutants studied. The sphingolipid species were also determined by mass spectrometry, and some differences were found in highly saturated sunflower mutants, which contained higher levels of GIPC, ceramides, and hydroxyl-ceramides.

KEYWORDS: Sphingolipids, sphingoid bases, sunflower mutant, *Helianthus annuus*, seed kernel

INTRODUCTION

Sphingoid bases are long-chain aliphatic derivatives carrying α- and γ-hydroxyl and β-amino groups.[1,2] They are synthesized by the condensation of palmitoyl-CoA and serine in a reaction that is catalyzed by the palmitoyl-CoA serine transferase.[3] The keto-derivative produced is reduced to yield a C18 sphinganine, which is the primary plant sphingolipid long-chain base (LCB) from which any other derivative can be generated by subsequent hydroxylation and desaturation steps. In plants, a large proportion of the LCBs are hydroxylated at the C4 position by sphingolipid hydroxylases, yielding 4-hydroxysphinganine or phytosphingosine.[4,5] The nomenclature used to name LCBs consists of a first letter that can be "d" for dihydroxylated bases or "t" for trihydroxylated ones, followed of two numbers separated by a colon, indicating the number of carbons and double bonds. Sphingolipid hydroxylation is important for these metabolites to be active.[6] Moreover, plant LCBs are often desaturated in the Δ4 or Δ8 position through the activity of specific desaturases that are involved in responses to certain kinds of abiotic stresses.[7] Free LCBs are found in very small amounts in plants, and they mostly exist in the form of complex sphingolipids such as ceramides and ceramide derivatives.[1,8]

Ceramides are LCBs bound to long-chain fatty acids through an amide bond to the β-amino group. They can also be bound to different polar groups in the α-hydroxy group, giving rise to a wide variety of chemical species (up to 300) that are the focus of modern sphingolipidomics.[9−11] The most common complex sphingolipid classes found in plants are glucosyl-ceramides (glucer), in which one or several saccharide units are bound to the 1-hydroxyl group of the long base,[12] and glucosyl inositol phosphoryl-ceramides (GIPCs); more polar derivatives with a phosphoinositol group between the ceramide core and the glycosyl units have been reported as membrane anchors of certain regulatory proteins.[13] The ceramide core can be formed by any of the LCBs that can be synthesized by plants and a 16−26 carbon atom fatty acid, which in plants are usually hydroxylated in the 2 (or α) position. In sphingolipid nomenclature, these hydroxylated fatty acids are distinguished from common ones by adding the letter "h" to their lipid number (i.e., h16:0). In general, only free ceramides display high proportions of nonhydroxylated fatty acids in their molecules.[14]

The sphingolipid composition of different plant species has been defined in some studies, and it depends on the variety of LCBs and fatty acids in each. Thus, while some species are rich in diunsaturated (d18:2) bases, like tomato or potato, these bases may be rare or even absent in other plants, depending of the presence of active Δ4-sphingolipid desaturases.[11,15]

Little is known about the contribution of sphingolipids in the human diet, so there is not any nutritional requirement regarding them. However, studies with experimental animals pointed to a possible role of these lipids in the inhibition of colon carcinogenesis and improvements of the low-density versus high-density lipoprotein cholesterol balance, which suggested that they are functional components of food.[16,17]

The LCB composition of different sunflower tissues has been reported previously.[18] Indeed, the LCB profile in this species is similar to that found in *Arabidopsis*, accumulating mainly trihydroxylated and Δ8-unsaturated LCBs with no diunsaturated derivatives. The proportion of the different species differs as a function of the tissue analyzed and according to the levels of the sphingolipid-modifying enzymes. Because the synthesis of sphingolipids and glycerolipids is related, the influence of altered fatty acid metabolism on the sphingolipid fraction of several

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sunflower mutants was studied. High-oleic,[19] high-stearic, and high-palmitic[20] oils accumulated in the seeds of the mutants studied, while the fatty acid composition of triacylglycerols was closely related to the polar glycerolipid fraction, which also displayed changes in the acyl moieties.[20] Here, we have studied whether the mutations involved affect the sphingolipid composition of the seed, as they affect enzymes of the central lipid synthetic pathway and include the ketoacyl−acyl carrier protein synthases, stearate desaturase or oleate desaturase. The total LCB content investigated in the four lines was not significantly affected during the period of oil accumulation. However, some differences were found in the relative amounts of GIPCs, ceramides, and hydroxylceramides in highly saturated mutants and in the glu-cer molecular species in the high-stearic mutant. The influence of fatty acid metabolism on sphingolipid synthesis and the impact of the mutations present in these mutants on sphingolipid metabolism are discussed in the view of these results.

MATERIALS AND METHODS

Plant Material. Sunflower plants were cultivated in growth chambers at 25/15 °C (day/night), with a 16 h photoperiod, a photon flux density of 300 μmol m^{-2} s^{-1}, and with fertirrigation lines. Plants from the standard CAS-6 sunflower line and plants with high-oleic (CAS-9), high-stearic (CAS-3), and high-palmitic (CAS-5) traits were used in this study.

LCB Extraction and Purification. Fresh sunflower seed tissue (approximately 0.1−0.25 g) was homogenized in 3 mL of 1,4-dioxane and 2 mL 10% of $Ba(OH)_2$ after the addition of 35 nmol of d17:$1^{\Delta 4t}$ as the internal standard. This mixture was digested at 110 °C for 24 h according to a modified version of the methods described by Sperling et al.[21] Once the tissue had been digested, sphingoid bases were extracted with 5 mL of chloroform, the fatty acid salts remained in the aqueous phase, and the LCBs were then recovered from the chloroform in their protonated form by extraction with 5 mL of HCl (0.4 M). The resulting aqueous extract was neutralized with 2 M KOH, and the fatty acid-free bases were again extracted with 5 mL of chloroform.[22] The organic solvent was then evaporated under nitrogen at 40 °C, and the resulting residue was resuspended in 0.1 mL of ethanol. LCBs were transformed into their *ortho*-phthalaldehyde (OPA) derivatives in a 15 min reaction with 100 μL of OPA reagent (25 mg of OPA and 25 μL of 2-mercaptoethanol dissolved in 0.5 mL of ethanol and made up to 50 mL with aqueous Na_2BO_3 [pH 10.5]) carried out at room temperature.[11] The resulting OPAs were made up to 1 mL with methanol and cleared by centrifugation. The cleared supernatants were then submitted to high-performance liquid chromatography (HPLC)−fluorescence analysis.

HPLC System. The HPLC system used in this work was a Waters 2695 separation module with a Waters X-Bridge 4.6 mm × 250 mm reversed-phase column and a Waters 2475 fluorescence detector. The OPA derivatives were separated using isocratic methanol/phosphate buffer 90:10 as the mobile phase at a flow of 1 mL/min. The excitation and emission wavelengths of the detector were set at 340/455, and the peaks were identified using commercial standards and on the basis of previous separations.[17] Assignation was later validated by HPLC-MS, and quantification was carried out by comparing the relative areas corresponding to each peak with that of the internal standard d17:$1^{\Delta 4t}$.

Sphingolipid Profiling. Developing sunflower endosperm tissue (50−100 mg) was ground in a glass homogenizer in the presence of 3 mL of the lower phase resulting from the mixture 2-propanol/water/hexane (55:25:20; solvent 1) and 10 μL of an internal standard mix that contained ovine ganglioside GM1 (100 nmol/mL), C12-glucosyl ceramide (50 nmol/mL), sphingosine (C17 base; 5 nmol/mL), and sphingosine-1-phosphate (5 nmol/mL) as described by Markham et al.[23]

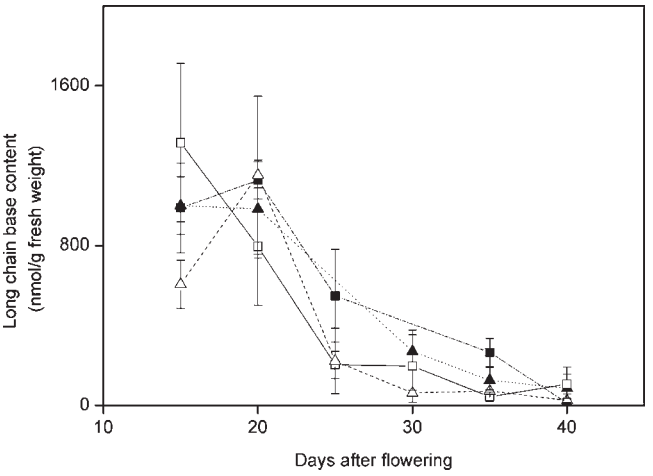

Figure 1. LCB content of seeds from different sunflower mutants at different stages of development. This study included common sunflower (CAS-6; ■, dashed dotted line), high-oleic mutant (CAS-9; ▲, dotted line), high-stearic mutant (CAS-3; □, dashed line), and high-palmitic mutant (CAS-5; △, solid line). Results are the average of at least three independent measurements.

The resulting extract was transferred to a clean glass centrifuge tube, and the homogenizer was then rinsed with an additional 3 mL of solvent 1. The combined extracts were heated at 60 °C for 15 min and spun at 500*g* for 10 min. The cleared supernatant was transferred to a clean tube, and the pellet was again extracted with an extra 3 mL of solvent 1 as above. The combined organic phases were dried under nitrogen at 60 °C, and the resulting residue was resuspended in 1.4 mL of 33% monomethyl amine in ethanol and incubated at 50 °C for 1 h. Subsequently, 0.6 mL of water was added, and the sample was vortexed until it was fully dissolved. After it was incubated for a further hour, it was analyzed by HPLC/ESI-MS/MS. Lipids were separated at 40 °C on a SUPELCOSIL ABZ+Plus column fitted with a guard cartridge. The binary gradient consisted of solvent A (THF/methanol/5 mM ammonium sulfate 3:2:5 by volume) and solvent B (THF/methanol/5 mM ammonium sulfate 7:2:5 by volume) both containing 0.1% formic acid, in the proportions reported by Markham and Jaworsky.[15] The different sphingolipid species were detected and characterized in a 4000 QTRAP LC/MS/MS system (ion spray voltage, 5000 V; source temperature, 650 °C; GS1 at 90 psi; GS2 at 50 psi; curtain gas at 20 psi; and interface heater on at 100 °C), and the data were collected with Analyst 1.4.2 software (Applied Biosystems) as described by Markham et al.[23]

Statistical Analysis. Statistical analysis was based on at least three independent determinations (three different preparations) as it is stated in the figure and table legends. All results were so expressed as averages ± standard deviations. All analysis was performed using the software OriginPro 8.0 software (OriginLab Corp., Northampton, United States) using a confidence level of 95%.

RESULTS

LCB Content of Developing Sunflower Seed Endosperm. The total LCB content in the developing sunflower seeds of all of the mutants was determined by HPLC over the entire period of seed development (Figure 1). All mutants studied displayed a similar profile, with high levels of sphingolipid accumulating at the beginning of seed development (15−20 days after flowering, DAF), declining progressively until they were hardly detectable in dried seeds (up to 40 DAF). The LCB composition of the different lines studied was also examined (Table 1), and in all

Table 1. Sphingolipid LCB Composition in Different Sunflower Mutants during Development[a]

	LCBs (mol %)			
	DAF			
species	15	20	30	40
		CAS-6		
t18:1$^{\Delta 8Z}$	3.7 ± 1.0	7.4 ± 0.2	6.9 ± 2.5	12.5 ± 1.5
t18:1$^{\Delta 8E}$	36.0 ± 14.4	56.7 ± 5.2	52.8 ± 11.4	22.6 ± 6.1
t18:0	3.6 ± 1.7	2.1 ± 0.7	1.2 ± 0.9	3.1 ± 1.1
d18:1$^{\Delta 8Z}$	2.0 ± 0.1	1.3 ± 0.2	2.9 ± 2.0	6.6 ± 3.5
d18:1$^{\Delta 8E}$	52.3 ± 6.8	30.3 ± 6.4	36.2 ± 12.5	55.2 ± 6.6
d18:0	2.3 ± 1.9	2.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
		CAS-9		
t18:1$^{\Delta 8Z}$	4.6 ± 0.6	6.3 ± 0.7	3.9 ± 3.7	3.4 ± 0.4
t18:1$^{\Delta 8E}$	49.9 ± 3.4	65.4 ± 13.3	36.9 ± 6.4	26.7 ± 2.8
t18:0	5.3 ± 0.6	3.0 ± 0.2	0.4 ± 0.3	0.0 ± 0.0
d18:1$^{\Delta 8Z}$	1.1 ± 0.28	0.7 ± 0.2	3.9 ± 0.6	14.3 ± 3.5
d18:1$^{\Delta 8E}$	35.8 ± 6.8	22.9 ± 5.8	54.7 ± 9.2	55.5 ± 6.6
d18:0	3.2 ± 0.2	1.7 ± 2.4	0.1 ± 0.1	0.0 ± 0.0
		CAS-5		
t18:1$^{\Delta 8Z}$	6.6 ± 1.7	8.3 ± 0.6	6.7 ± 3.8	9.4 ± 9.9
t18:1$^{\Delta 8E}$	49.1 ± 1.3	66.3 ± 17.2	52.8 ± 12.0	39.9 ± 15.7
t18:0	3.9 ± 0.2	1.9 ± 1.2	0.5 ± 0.3	0.0 ± 0.0
d18:1$^{\Delta 8Z}$	0.1 ± 0.2	0.3 ± 0.2	0.9 ± 0.4	1.1 ± 1.6
d18:1$^{\Delta 8E}$	39.5 ± 2.8	23.3 ± 15.9	38.9 ± 13.6	49.5 ± 7.5
d18:0	0.7 ± 0.1	0.1 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
		CAS-3		
t18:1$^{\Delta 8Z}$	3.5 ± 1.6	4.1 ± 0.4	4.9 ± 1.5	13.2 ± 8.8
t18:1$^{\Delta 8E}$	25.8 ± 17.7	9.3 ± 2.0	79.2 ± 2.6	38.6 ± 13.3
t18:0	2.8 ± 2.0	3.8 ± 0.9	2.1 ± 0.8	0.9 ± 0.9
d18:1$^{\Delta 8Z}$	1.8 ± 0.9	0.8 ± 0.2	0.5 ± 0.0	3.2 ± 1.2
d18:1$^{\Delta 8E}$	60.8 ± 6.8	41.8 ± 3.4	13.4 ± 0.2	44.0 ± 6.6
d18:0	5.3 ± 1.9	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.2

[a] CAS-6, common sunflower; CAS-9, high-oleic sunflower; CAS-5, high-palmitic sunflower; and CAS-3, high-stearic sunflower. The results are the average of at least three independent measurements.

cases, only monosaturated bases were found, and no Δ4 bases were evident. However, the degree of LCB hydroxylation displayed some dependence on the developmental stage. Thus, it seemed that trihydoxylated bases increased from 15 to 20 DAF, to reach a maximum at this time, and then decreased at later stages of development. This profile was different in the mutant CAS-3, which reached the maximum content of trihydroxylated at 30 days after anthesis. The content and compositions of the LCBs analyzed were in general subjected to high variability.

Sphingolipid Species in Developing Sunflower Seeds. The most prominent sphingolipid species present in seeds were quantified and determined in an analytical MS study carried out on the different sunflower mutants. The predominant sphingolipid classes in seeds from 15 to 20 DAF, the period with the highest LCB content, were glu-cer, GIPCs, ceramides, and hydroxyl-ceramides (Figure 2). The most abundant class were the glu-cer, ranging from 210 to 260 nmol/g FW, with no significant differences in its content among the mutants studied.

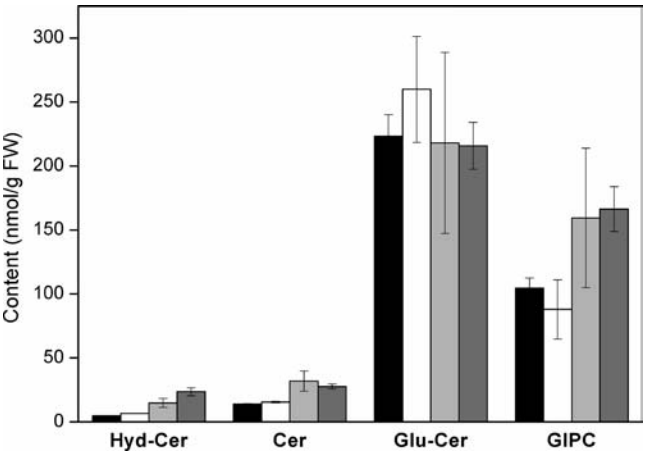

Figure 2. Sphingolipid content in developing seeds from different sunflower mutants. This study included common sunflower (CAS-6; black), high-oleic mutant (CAS-9; white), high-stearic mutant (CAS-3; light gray), and high-palmitic mutant (CAS-5; dark gray). The results are the average of four independent measurements. Seeds were harvested between 15 and 20 DAF.

The GIPC levels showed higher variation across mutants with a tendency to increase for lines containing higher levels of saturated fatty acids, CAS-3, and CAS-5. Ceramides and hydroxyl-ceramides were present in lower amounts in sunflower seeds, and again, they were slightly more prominent in highly saturated mutants.

Sphingolipid Profiling in Developing Seeds of Different Sunflower Mutants. The study on sunflower sphingolipids was completed by investigating the species present in the different sphingolipid classes (Figures 3−6 show the composition in each mutant). The predominant GIPC species carried trihydroxylated long bases bound to α-hydroxylated long-chain fatty acids, which were predominantly saturated C22−C26 fatty acids. Very few monosaturated fatty acids were found in these species. The C16 hydroxylated fatty acids were appreciable in all sphingolipid species, in contrast with the very low amounts of C18 fatty acids, typically the most abundant in glycerolipids. When the composition in seeds with altered fatty acid metabolism was compared, all of the profiles were similar other than that of the high-stearic CAS-3 mutant seeds. In this case, an important proportion of the GIPCs contained the d18:1-h16:0 pair, at the expense of the trihydroxylated LCBs bound to long-chain fatty acid moieties. The most abundant sphingolipid species accumulated by seeds was glu-cer. In all cases, the molecular species of glu-cer were different to those present in the GIPC fraction, and the most common species found contained the d18:1-h16:0 pair in all of the lines studied. Ceramides and hydroxyl ceramides displayed a higher content of trihydroxylated bases and very long-chain fatty acid moieties in their predominant species. In the case of hydroxyl ceramides, species containing a C16 fatty acid were more abundant than in nonhydroxylated derivatives. Again, no significant differences were found among the different mutants investigated.

DISCUSSION

There is large structural diversity among plant sphingolipids, important components of regulatory and signaling pathways.[24,25] The variety of different possible ceramides, in addition to the

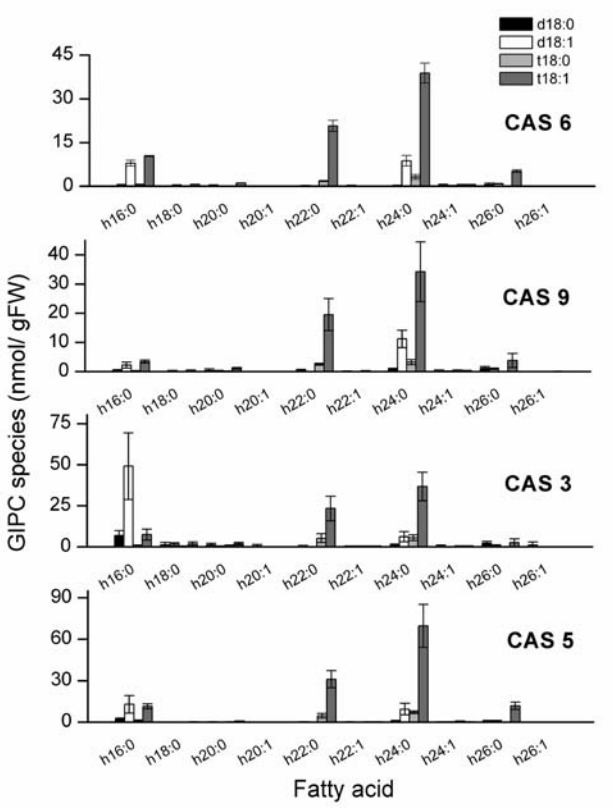

Figure 3. Glucosyl inositol phosphoryl-ceramide species in developing seeds from different sunflower mutants. This study was performed on the common sunflower (CAS-6) and on the high-oleic (CAS-9), high-stearic mutant (CAS-3), and high-palmitic mutants (CAS-5). Seeds were harvested between 15 and 20 DAF, and the results are the average of four independent measurements.

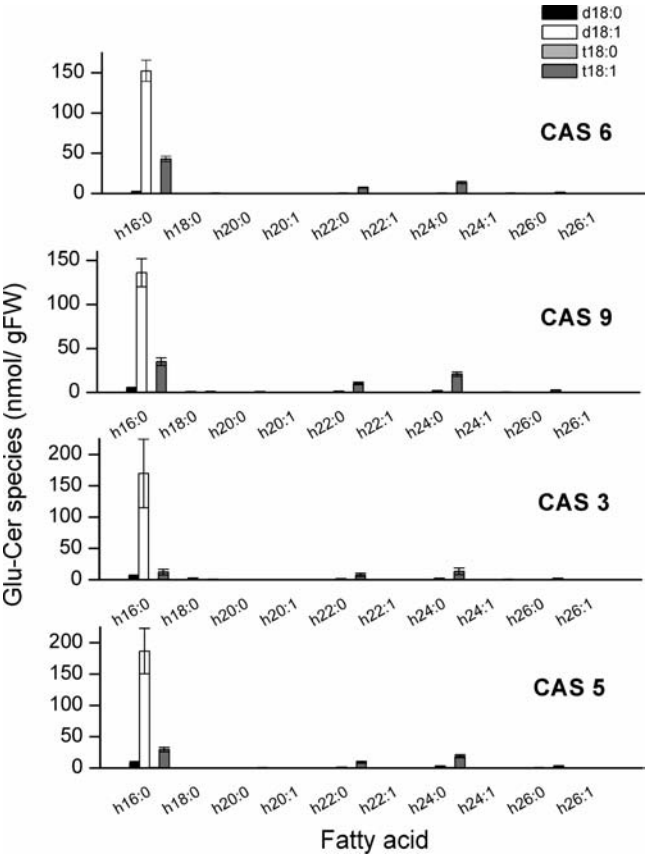

Figure 4. Glucosyl ceramide species in developing seeds from different sunflower mutants. This study was performed on the common sunflower (CAS-6) and on the high-oleic (CAS-9), high-stearic mutant (CAS-3), and high-palmitic mutants (CAS-5). Seeds were harvested between 15 and 20 DAF, and the results are the average of four independent measurements.

possible combinations of saccharide units in the polar moiety, means that a very large number of possible complex sphingolipid species can be found in plants. These sphingolipid ceramide moieties are synthesized by mechanisms closely related to those that lead to the synthesis of glycerolipids. The ceramide moieties present in a plant change as a function of the sphingolipid species, although it is not clear if they respond to changes in fatty acid metabolism. Several sunflower mutants have been produced by altering the pathway of fatty acid synthesis, and their oils have different fatty acid compositions. These mutants had depleted or deficient oleate desaturase, stearate desaturase, and fatty acid synthase II activities, producing high-oleic, high-stearic, and high-palmitic phenotypes, respectively.[19,26,27] These alterations only influenced seed fatty acids, affecting both triacylglycerols and polar membrane lipids.[20,28] The alterations in the pathways of synthesis of fatty acids changed the size and composition of the acyl-CoA pools, which could alter sphingolipid synthesis and induce variations in the composition of these lipids. Indeed, palmitic acid export from plastids is restricted in the *fatB Arabidopsis* knockout mutant,[22] which is deficient in cytosolic palmitoyl-CoA. When the LCB composition of these plants was analyzed, no changes were evident when compared with control plants. However, it was then speculated that the dwarf phenotype of these plants might be caused by a deficiency in sphingolipid synthesis based on the shortage of palmitoyl-CoA, as occurs in *Arabidopsis* plants with reduced LCB synthesis.[10]

The LCB composition of different sunflower tissues has been determined previously,[18] concluding that the major components of this species contain d18:1$^{\Delta 8}$ and t18:1$^{\Delta 8}$, with only minor amounts of the saturated homologues.

The degree of hydroxylation changes as a function of the tissue and the developmental stage, as determined by the amounts of the different sphingolipid hydroxylases expressed. Here, the total amount and composition of LCBs in the seeds of different sunflower mutants were analyzed during the whole period of development, which can be divided into three different stages as in other oil crops.[29] The first stage involves embryo formation and rapid cell division (15−20 DAF). These cells then expand and accumulate most oil during the intermediate stage (20−30 DAF). Finally, in the last period of development from 30 to 50 DAF, biosynthetic pathways are switched off, and seed drying occurs. Sunflower seeds accumulate the most LCBs in the first stage of development, accounting for up to 1 μmol of LCBs per g fresh weight (Figure 1). The LCB content of sphingolipids decreased during the period of oil accumulation and seed drying, reaching a minimum in dry seeds.

The total amount of LCBs did not vary as a function of the mutant, which indicates that the synthesis of these metabolites was not strongly influenced by the changes in fatty acid metabolism that occurred in these mutants (Figure 1). The LCB composition of the different mutants was also studied, and as

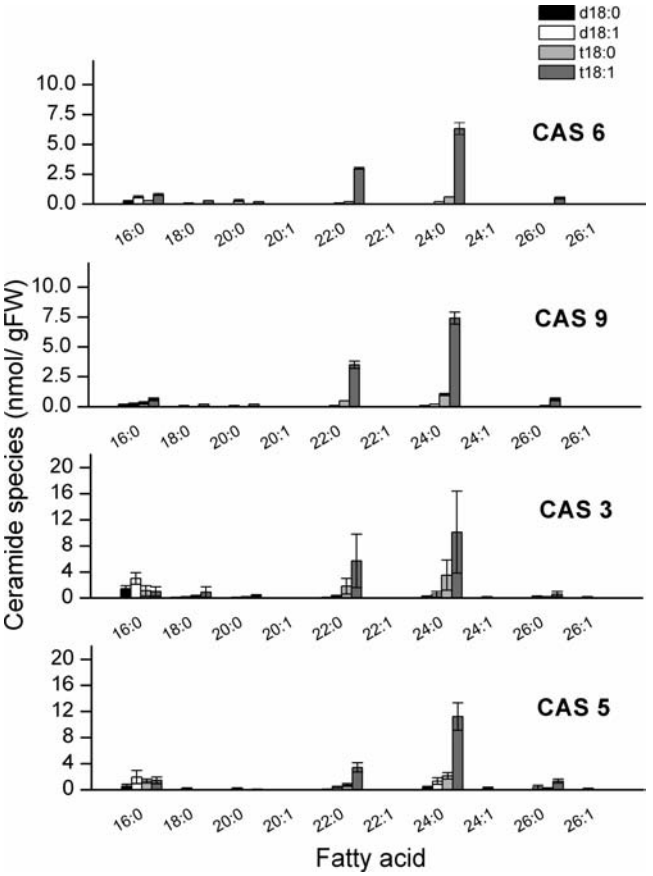

Figure 5. Ceramide species in developing seeds from different sunflower mutants. This study was performed on the common sunflower (CAS-6) and on the high-oleic (CAS-9), high-stearic mutant (CAS-3), and high-palmitic mutants (CAS-5). Seeds were harvested between 15 and 20 DAF, and the results are the average of four independent measurements.

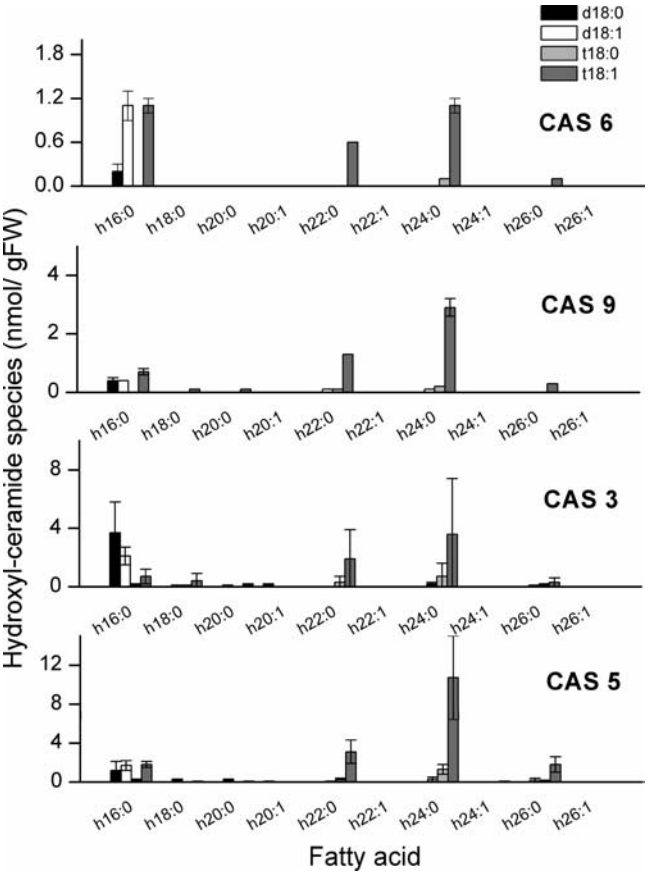

Figure 6. Hydroxyl ceramide species in developing seeds from different sunflower mutants. This study was performed on the common sunflower (CAS-6) and on the high-oleic (CAS-9), high-stearic mutant (CAS-3), and high-palmitic mutants (CAS-5). The seeds were harvested between 15 and 20 DAF, and the results are the average of four independent measurements.

reported previously, sunflower seeds only have Δ8 monounsaturated bases and smaller amounts of saturated ones, the latter displaying different degrees of hydroxylation as a function of the developmental stage (Table 1). Thus, at early stages (15 DAF), there were more dihydroxylated bases, which tended to decrease at the expense of trihydroxylated bases, which peaked at 20 DAF in all lines except the CAS-3 mutant, in which they reached the maximum at later stages of development (30 DAF). These changes were probably caused by differential activation of the corresponding sphingolipid hydroxylases as indicated elsewhere.[18] Furthermore, it was also clear that the glycerolipid fatty acid composition of the different lines did not alter the LCB composition of the seeds.

This study was completed by profiling the different sphingolipid classes and species in the developing seeds of the mutants at early stages of development (15−20 DAF), when the sphingolipid content was the highest. The main sphingolipid species in these plant tissues were quantified, and the most abundant sphingolipid class in developing sunflower seeds was glu-cer, as in other oilseeds like soybean (Figure 2). GIPCs are highly polar sphingolipids, which makes them difficult to extract as lipids using most common extraction protocols, although they were also major components of the sphingolipid fraction in developing sunflower seeds. There were differences in the sphingolipids among the mutants, whereby sunflower lines with a high saturated fatty acid content tended to contain more GIPCs, although the differences were not statistically significant based on the results in Figure 2. The ceramide and hydroxylceramide contents displayed similar tendency as the GIPCs, with more of these lipid classes accumulating in highly saturated mutants. These results suggest that fatty acid composition can slightly affect the classes of sphingolipids that are present in sunflower lines with a higher saturated fatty acid content, which could be related with the higher levels of saturated acyl-CoAs in the cytosolic pools of these mutants. These saturated acyl-CoAs are substrates for the sunflower fatty acid elongase enzymes,[30] which produce the very long-chain fatty acids typically found bound to LCBs in the ceramide core of most sphingolipid species.

When the different ceramide species present in the sphingolipid classes from sunflower seeds were analyzed, glu-cer ceramides mainly contained dihydroxylated bases bound to α-hydroxyl palmitate (Figure 4). The fatty acid moiety of this sphingolipid species was highly hydroxylated, like in GIPCs. Moreover, this hydroxylation of the C2 position (α-hydroxylation) does not occur on the free fatty acid or on acyl-CoA esters but, rather, on the fatty acids already bound to the ceramide core of the different sphingolipids, distinguishing the fatty acids within the sphingolipid fraction from those bound to

glycerolipids.[31] The composition of ceramides from the glu-cer fraction did not change from one mutant to another, and it was similar to those reported for oil seeds species (Figure 4). Nevertheless, the composition of the ceramide moieties in GIPCs was different to that found in glu-cer. Thus, there was a predominance of trihydroxylated bases bound to α-hydroxylated long-chain fatty acids, mostly h22:0 and h24:0, with very little h16:0 and h26:0 (Figure 3). This has been reported for other plants,[32] and it agrees well with the sunflower glycerolipid composition and the characteristics of sunflower fatty acid elongases, which were only active with saturated acyl-CoAs when assayed in vitro.[30] The sunflower seed glu-cer fraction contains high levels of d18:1 LCB, resembling that of soybeans[33] and differing from the cerebrosides in *Brassicaceae* that were rich in t18:1 base.[34] This result indicated that the enzymatic machinery synthesizing the different sphingolipid species was specific to different ceramide substrates. As a function of the ceramide composition of GIPCs in the different mutants, it was noteworthy that the high-stearic CAS-3 line accumulated a large amount of ceramide species carrying the d18:1h16:0 pair, more characteristic of the glu-cer sphingolipid class (Figure 3). This result is difficult to explain on the basis of the fatty acids in the CAS-3 line (a mutant containing high levels of stearic acid on a high-linoleic background), and it seemed more like an alteration in sphingolipid metabolism irrespective of the fatty acid synthetic pathway.

In all cases, the free ceramide and hydroxyl ceramide fractions displayed more t18:1 LCBs bound to very long-chain fatty acid species, with ceramide core compositions similar to those found in GIPCs. In this case, no clear differences were found among the different mutants investigated, and they were subject to wider variation (Figures 5 and 6).

The levels of sphingolipids of sunflower dry seeds were similar to those reported for other seeds or seed products. However, the levels of these lipids were much higher in immature seeds. The works studying the impact of sphingolipids on nutrition focused mainly in their total amounts but not in the LCB or fatty acid composition,[17] so no conclusions can be reached on the nutritional value of the sphingolipids from the different mutants.

In conclusion, sunflower seeds are a rich source of sphingolipids in their initial stages of development. Sunflower LCBs consisted of saturated and Δ8-monounsaturated derivatives with different proportions of di- or trihydroxylated species as a function of the developmental stage. Alterations in the seed glycerolipid composition did not affect the final LCB content or composition. However, an effect was observed when quantifying the different sphingolipid classes, suggesting that highly saturated CAS-5 and CAS-3 mutants generate slightly more GIPCs, ceramides, and hydroxylceramides. Other significant alterations were also found in the composition of the GIPCs in CAS-3, although these differences could not be associated with the changes that take place in fatty acid metabolism in this mutant.

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