RESEARCH

Day–Night Variation in Fatty Acids and Lipids Biosynthesis in Sunflower Seeds

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ABSTRACT

It is well known that fatty acid biosynthesis in leaves is closely related to photosynthesis; although a residual synthesis is also detected at night. But less known is the effect of the day–night cycle in fatty acid biosynthesis in heterotrophic tissues such as developing seeds. Through the use of radioactive precursors, we detected variations in the metabolism of fatty acids and lipids during the day–night period in sunflower (*Helianthus annuus* L.) seeds. These oscillations have been observed both in the fatty acids bound to triacylglycerols and in polar lipids, indicating a fine regulation of the lipids biosynthetic activities in sunflower developing seeds. Additionally, in the triacylglycerol fraction, periodic variations were observed in the oleic and linoleic acids content as a consequence of qualitative variations in the biosynthetic machinery during the day–night period related to temperature changes. Analyzing the rate of oleic acid desaturation, we observed a significant increase in activity in the middle of the night, indicative of the importance of nocturnal desaturation. Similar variations were also observed in the stearoyl-acyl carrier protein (ACP) desaturase activity. This study has shown the variation in the lipid biosynthetic capacity in sunflower developing seeds during the day–night cycle—including control over individual enzymes such as the stearoyl-ACP desaturase—and the influence of diurnal and nocturnal temperatures in the quality (oleic/linoleic ratio) of the oil.

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Abbreviations: ACP, acyl carrier protein; DTT, dithiothreitol; FAS, fatty acid synthetase; ODS, oleoyl phosphatidylcholine desaturase; SAD, stearoyl-ACP desaturase; TAG, triacylglycerol; TLC, thin layer chromatography.

In virtually all organisms, many aspects of behavior, physiology, and biochemistry change during the day. The photosynthetic carbon fixation in leaves during the day maintains the synthesis of sucrose and its export to the rest of the plant to be used in metabolism, storage, and growth. By contrast, during the night, the plant becomes a net carbon consumer. Part of the photosynthate is stored as starch in the leaves during the day and reused at night (Geiger et al., 2000). Some of these changes occur only in response to environmental stimuli, such as the light–dark cycles, while others persist in the absence of environmental changes. The biological rhythms that occur with a periodicity of approximately one day are known as circadian rhythms, one example of which is the adjustment of a plant's growth and development to the daily light–dark cycles (Somers, 1999; Yanovsky and Kay, 2001). This adaptation permits the plant to maintain the rhythm of many physiological processes and to anticipate the different cyclical events that occur in their environment, in this way providing it with an adaptive advantage (Harmer et al., 2000; Staiger, 2002). This phenomenon has been highlighted in the plant model *Arabidopsis thaliana*, in which rhythmic expression was identified in approximately 6% of the 8200 genes assayed in

Published in Crop Sci. 48:1952–1957 (2008).
doi: 10.2135/cropsci2007.11.0645

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microarrays, some of which are genes involved in lipid synthesis, principally of desaturases (Harmer et al., 2000; Schaffer et al., 2001; Ruuska et al., 2002).

In oilseeds, the synthesis of fatty acids up to oleic acid is performed within organelles known as plastids. In these organelles, a conjunction of enzymes denominated fatty acid synthase (FAS) successively lengthen the acyl skeleton by two carbons until forming palmitoyl-acyl carrier protein (ACP) and stearoyl-ACP, the latter being desaturated by the stearoyl-ACP desaturase (SAD) to form oleoyl-ACP. These acyl-ACPs must be hydrolyzed by the acyl-ACP thioesterases to exit into the cytoplasm and be used in the synthesis of triacylglycerols (TAGs). Once in the cytosol, the oleoyl phosphatidylcholine desaturase (ODS) can desaturate oleic acid, generating linoleic acid. Finally, the products formed in the endoplasmic reticulum serve as substrates for the synthesis of phospholipids, TAGs, and other neutral lipids (Harwood, 1996). The enzymes mentioned here are of vital importance to define the physical-chemical properties of the reserve lipids, given that their activity and affinity for the distinct substrates determine both the composition and the final content of seed lipids.

Fatty acid biosynthesis in leaves and green seeds, such as soybeans [*Glycine max* (L.) Merr.], is almost entirely dependent on light (Browse et al., 1981; Ohlrogge, 1997). But some synthesis occurs in the dark, representing about 12 to 20% of that produced under light conditions. To date, little attention has been paid to the day–night variations in heterotrophic tissues such as seeds, most studies focusing on green tissues, leaves and green seeds, when studying this type of behavior (Browse et al., 1981; Ciceri et al., 1999; Ekman et al., 2007). In this work, we have examined the liposynthetic metabolism of developing sunflower (*Helianthus annuus* L.) seeds in vivo during the day–night period, administering radioactive precursors to identify possible variations during this biosynthetic process.

MATERIALS AND METHODS

Plant Material

The sunflower seeds used in this work were obtained from the standard CAS-6 public line and the cultivar Paraíso 30 (Nidera Seeds, Buenos Aires, Argentina); both genotypes have standard composition of fatty acids (high linoleic acid) into the oil at maturity. The CAS-6 plants were cultivated in growth chambers at 25/15°C (day/night) temperature, with a 16-h photoperiod and at a light intensity of 300 μmol m^{-2} s^{-1}. Seed samples were collected at 15 d after flowering for analysis, sampling over 2 or 4 d depending on the experiment. The hybrid Paraíso 30 was cultivated in the field after late spring sowing in Buenos Aires, Argentina (34°35′ S, 58°29′ W), where the average temperature from flowering to physiological maturity was 30/20°C (day/night), with a 13-h photoperiod and 2020 μmol m^{-2} s^{-1} of photosynthetic photon flux density. Seed samples were collected at 15 d after flowering, but because of the greater temperature in the field than in the chamber, this age correspond to an older state of seed development in respect to plants cultivated in the chamber. The developmental age of seeds, expressed in a thermal time accounting for calendar time and temperature of growing, with 6°C as base temperature (Connor and Hall, 1997), was 235 and 380°Cd by chamber and field experiments, respectively. For each experiment, seeds from the external flower rings of three different plants were sampled.

Incubation with Sodium [2-^{14}C]Acetate

Achenes were collected from the capitulum immediately before incubating them with [2-^{14}C]acetate, and five to six seeds (achenes without hull and seed coat) were used for each assay. The fresh weight was recorded, and the seeds were sliced into three equal-sized parts and placed into a 10-mL glass tube. Radioactive acetate assays were performed as described previously (Singh et al., 1986) with some modifications. The seeds were incubated in the glass tubes in 300 μL of 50 mM MES buffer pH 6.0 (2-[N-morpholino] ethanesulfonic acid; Sigma-Aldrich, Steinheim, Germany), and the assay was initiated by adding 200 μL of sodium [2-^{14}C]acetate in 0.3 mM MES buffer pH 6.0 (specific activity 2.11 GBq $mmol^{-1}$; Amersham-Pharmacia, Buckinghamshire, UK). The seeds were incubated with agitation at 20°C for 4 h, and the assay was terminated by immersing the tubes in an 80°C water bath before washing the seeds three times in water.

Lipid Analysis

Lipids were extracted (Hara and Radin, 1978) after grinding the seeds with a pestle and sand in a screw-cap glass tube (10 by 13 mm). The total lipids were resuspended in 1 mL of hexane/isopropanol (7/2, v/v), and the radioactivity incorporated to the lipid fraction was measured in a scintillation counter (Rackbeta II; LKB, Sweden).

Fatty acid methyl esters were prepared by treating the sunflower cotyledons or lipid sample with 3 mL of methanol/toluene/sulfuric acid (88/10/2, v/v/v) for 1 h at 80°C (Garcés and Mancha, 1993). After cooling, fatty acid methyl esters were extracted twice with 2 mL of heptane and analyzed by gas-liquid chromatography on an SP-2380 capillary column of fused silica (Supelco, Bellefonte, PA; 30 m length, 0.25 mm i.d., 0.20 μm film thickness;). The carrier gas was hydrogen at a flow rate of 28 cm s^{-1}. The detector and injector temperatures were 200°C, whereas the oven temperature was 170°C. Different methyl esters were identified by comparison with known standards.

The triacylglycerol and polar lipid fractions were separated by thin layer chromatography (TLC) (Merck, Darmstadt, Germany), and the plates were developed with hexane:diethyl ether:formic acid (75/25/1, v/v/v). Fatty acid methyl esters were prepared as described above, and after cooling, fatty acid methyl esters were extracted twice with 2 mL of heptane. To isolate the fatty acids on the basis of chain length, methyl esters were separated by reverse-phase TLC on silica gel plates previously coated with 2.5% vaseline oil (Panreac, Barcelona, Spain) in hexane, using acetonitrile:hexane (90/10, v/v.) as the solvent. The methyl esters of palmitic and oleic acid have the same retention factor in these plates, and they were therefore scraped off and separated on $AgNO_3$–TLC plates (10% by weight in acetonitrile) using hexane:diethyl ether (85/15, v/v) as the mobile phase. The different radioactive lipid classes and fatty acid methyl esters were detected and quantified using a two-dimensional autoradiography

scanner (Instant Imager, Packard, Canberra), and they were identified by comparison with known standards.

Stearoyl-ACP Desaturase Assay

Labeled stearoyl-ACP substrate was prepared using a recombinant acyl-ACP synthetase from *Escherichia coli*, kindly provided by John Shanklin (Brookhaven National Laboratory, Upton, NY). Acylation reactions contained 50 μg of recombinant spinach ACP-I (Sigma), 660 MBq (~0.1 μmol) of [1-^{14}C] fatty acid ammonium salt, 5 mM ATP, 2 mM dithiothreitol (DTT), 4 mM $LiCl_2$, 10 mM $MgCl_2$, 100 mM Tris (pH 8.0), and 10 μg acyl-ACP synthetase in a final volume of 0.5 mL. Reactions were performed at room temperature for 3 to 4 h, and the stearoyl-ACP was purified and concentrated by ion exchange chromatography on DEAE-sepharose (Rock and Garwin, 1974).

Typically, 100 mg of developing sunflower cotyledons was ground in 1 mL of 50 mM Tris (pH 8.0) and 5 mM DTT using an ice cooled glass homogeniser. The resulting homogenate was centrifuged for 10 min at 10,000 *g*, and the clear supernatant was used for enzyme assays. Protein content was quantified by a colorimetric method (Bradford, 1976) using bovine gamma globulin as the standard. Stearoyl-ACP desaturase (EC 1.12.99.6) activity was assayed in the soluble fraction of sunflower cotyledons (Cahoon et al., 1994) with some modifications; the assay medium contained 33 mM PIPES pH 6.0, 3.3 M ascorbate, 0.7 mM DTT, 8000 U catalase, 5 μg bovine serum albumin, 20 μg spinach ferredoxin (Sigma), 80 mU spinach ferredoxin-NADP reductase (Sigma), 1.25mM NADPH, 0.72 MBq of [1-^{14}C]stearoyl-ACP, and 1 to 30 μg of soluble protein in a final volume of 0.15 mL. Assays were run long enough to convert approximately 10% of the initial substrate (10–30 min) before terminating and saponifying the products with 0.85 mL of 2.35 M NaOH at 80°C for 30 min. After saponification, the assay medium was acidified with 0.35 mL of H_2SO_4, and free fatty acids were extracted three times with 2 mL of hexane. The organic phases were combined and evaporated under nitrogen. Free fatty acids were then methylated for 1 h at 80°C in 1 mL 2.5% methanolic sulfuric acid, and 3 mL of 5% NaCl was added before the methyl esters were extracted three times with 2 mL hexane. The solvent was again evaporated under nitrogen and the resulting lipid extract fractionated by argentation TLC. The different fatty acid methyl ester radioactivity spots in the TLC were detected and quantified using a two-dimensional autoradiography scanner (Instant Imager, Packard, Canberra), and they were identified by comparison with known standards.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-Acetate into Lipids during Light–Dark Cycles

In plants grown in chambers, the incorporation of radioactivity into the total lipid, TAG, and polar lipid fractions fluctuated during the light–dark cycles, displaying two peaks: diurnal and nocturnal (Fig. 1A). The majority of the radioactivity was incorporated in the middle of the light period (first peak), while there was less incorporated during the period of darkness for the three lipid fractions analyzed (second peak). Furthermore, due to the developmental stage of the seeds, the synthesis of lipids augmented from 1 d to the next. This growth was reflected in the increased incorporation of radiolabel between the first and second day of the experiment (Fig. 1A). These results are in accordance with results obtained previously when corn (*Zea mays* L.) leaves and discs of spinach leaves (*Spinacia oleracea* L.) were

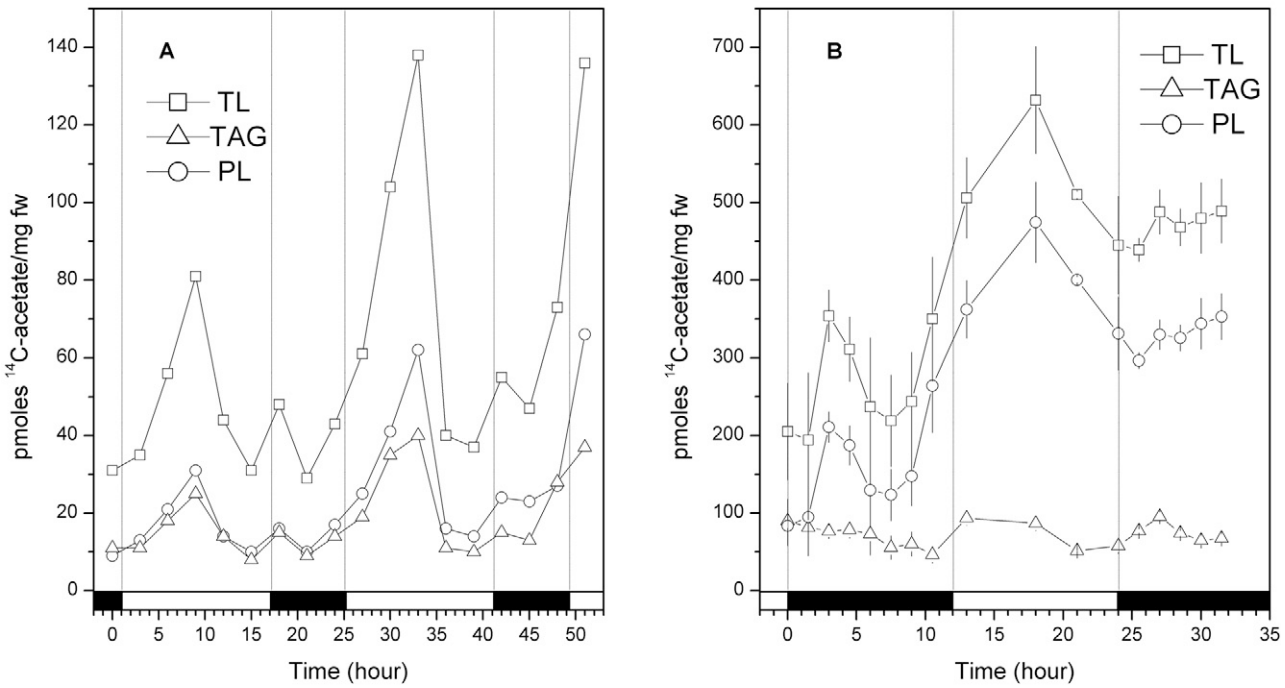

Figure 1. Incorporation of ^{14}C-acetate into lipids of developing sunflower seeds during the light–dark cycles. (A) Plants of the CAS-6 inbred line grown in culture chambers. (B) Plants of the 'Paraíso 30' hybrid grown in the field. The radioactivity present in the organic fractions of total lipids (TL) is shown: (TL, □), triacylglycerols (TAG, ○), and polar lipids (PL, △); diurnal period (*x* axis, white bar) and nocturnal period (black bar). The results shown are from one plant in A and are the mean ± SE of three plants in B. FW, fresh weight.

incubated in ^{14}C-acetate (Browse et al., 1981). In both these cases, the majority of lipid synthesis occurred in the presence of light, and that which was observed in the dark was never greater than 15% of that in the light period.

When the same assay was performed using sunflower seeds from plants grown in the field, a similar pattern was observed, although the environmental conditions, including the photoperiod, were distinct (Fig. 1B). The incorporation of radioactivity into the organic fraction also proved to be greater in the middle of the day and less during the nocturnal period, but both incorporation peaks, nocturnal and diurnal, were observed. In contrast to plants of the similar age cultivated in chambers, the plants grown in the field displayed a wider oscillatory phase. Indeed, the relative maximum at the end of the day or the onset of the dark phase was clearer in these plants than that observed in the plants grown in chambers.

On the other hand, the seeds from plants grown in the field channeled a greater quantity of the ^{14}C-acetate absorbed to the TAG fraction than the plants grown in culture chambers. In the sunflower, as a measure of how seed filling advances, the flux of carbon directed toward lipids is principally destined to reserve lipids and not to structural lipids. Thus, the majority of radioactivity found in the TAG of seeds from plants grown in the field is that which is expected for a more advanced physiological state.

Oscillations in Lipid Labeling under Constant Conditions

Given that the sunflower seeds' ^{14}C-acetate incorporation into the lipid fraction increased even before the light period commenced (Fig. 1), we wanted to study whether this behavior was reflected in the existence of an internal rhythm independent of the light–dark cycles. To achieve this, after 2 d of normal light–dark cycles, the plants were maintained in conditions of continuous light and temperature, and subsequently, the distribution of the radioactivity incorporated into the lipid fraction of the developing seeds was analyzed (Fig. 2). During the light–dark cycles, the incorporation of ^{14}C-acetate into the lipid fraction maintained the same oscillations and duration. However, when the plants were passed to conditions of constant light and temperature, the same oscillations in incorporation were maintained but the periodicity decreased. As such, while the incorporation of radioactivity was greater during the day and less during the subjective night, the periodicity of the oscillations passed from 24 to 30 h while their amplitude diminished. This increment in the periodicity of oscillation in continuous light was distinct to the behavior observed in other biological rhythms of plants. For examples, it has been demonstrated that when *Arabidopsis* plants pass to continuous light, the periodicity of the oscillations in CAB:LUC reporter gene expression shortens (Millar et al., 1995).

In contrast to other types of seeds that possess photosynthetic activity, green seeds, such as those of soybean, *Arabidopsis* or rapeseed (*Brassica napus*), the sunflower seed is a real nongreen heterotrophic seed (Alonso et al., 2007). This implies that the synthesis of reserve products, proteins and oil, depends exclusively on the supply of fixed carbon in the form of sugars from photosynthesis in the leaves. Photosynthesis in the leaves produces an excess of assimilated products, part of which is stored in the leaf as starch and another part of which is distributed through the phloem in the form of sucrose to maintain the growth of heterotrophic tissues such as the roots, reproductive organs, and seeds. The peaks of oil synthesis observed probably correspond to the diurnal utilization of sucrose produced directly by photosynthesis (the absolute maximum) and the relative nocturnal maximum to the nocturnal use of the sucrose that is formed from the degradation of the starch accumulated during the day in the leaves (Chia et al., 2004).

Recently, the behavior of the pools of starch and sucrose in conditions of continuous light during 4 d was described for *A. thaliana* leaves (Lu et al., 2005). These results demonstrated that the starch content diminished around the subjective nights with intervals of about 16 and 32 h and that sucrose cycled with a less solid pattern but with a similar variation in the interval. These data further indicate that

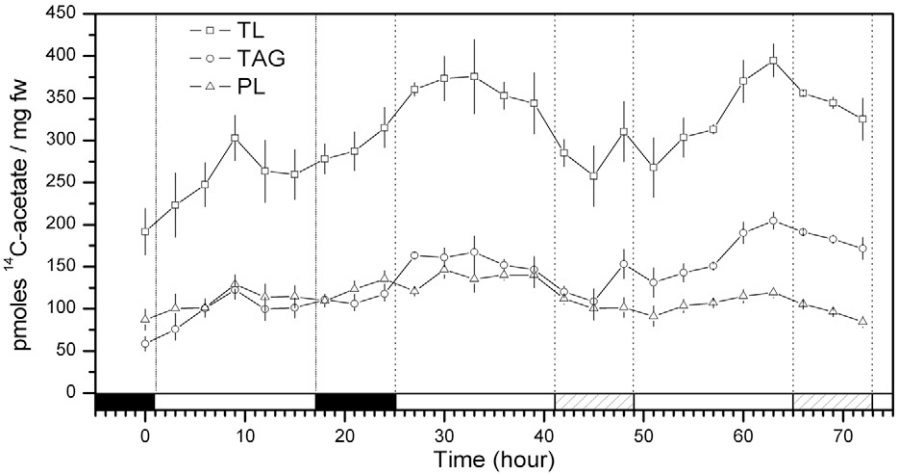

Figure 2. Oscillations in the labeling of lipids under conditions of constant light and temperature. Plants of the CAS-6 sunflower line grown in culture chambers, 25/15°C, 16-h photoperiod, and 300 μmol m^{-2} s^{-1}, were switched to conditions of continuous light and temperature (25°C). The seeds were sampled every 3 h and incubated for 3 h with ^{14}C-acetate before extracting the organic fraction. The radioactivity present in the organic fractions of total lipids (TL) is shown: (TL, □), triacylglycerols (TAG, ○) and polar lipids (△); diurnal period (*x* axis, white bar), nocturnal period (black bar), and subjective night (hatch-marked bar). Values are the mean ± 1 SE from three different plants. FW, fresh weight.

cycles of lipid synthesis are maintained in the seed depending on the supply of sucrose and suggest that the amplitude of the cycle suffers a decrease due to the fact that this supply is more constant.

Diurnal Variations in the Fatty Acid Composition of Seed Lipids

To study whether the fluctuations observed in the labeling of de novo synthesized fatty acids had repercussions on the properties of the oil during the light–dark cycle, we determined the fatty acid composition of the oil of seeds sampled from sunflower plants at 3-h intervals (Fig. 3). During the light–dark cycles, the stearic and palmitic acids content did not oscillate, and these fatty acids were maintained as stable. In contrast, the oleic and linoleic content did oscillate with the light–dark cycles. Indeed, the oleic acid content increased during the day before decreasing at the onset of the night, at the expense of linoleic acid synthesis, of which it is a precursor. However, the alternating behavior of both oleic and linoleic acid, disappears once under constant light conditions. In sunflower seeds, temperature affects the activity of the ODS responsible for the synthesis of linoleic acid (Garcés et al., 1992). The temperature may regulate this enzyme both by altering its expression and by regulating its activity since it is an enzyme that can be thermally inactivated (Garcés et al., 1992; Sarmiento et al., 1998). The increment of SAD activity in constant conditions of light and temperature (Fig. 4), coupled with the inactivation of the ODS enzyme, favors the synthesis and accumulation of oleic acid in the oil. Likewise, our data confirm the nocturnal desaturation of oleic acid and the influence that the temperature during this period exerts on the activity (Izquierdo et al., 2002; Izquierdo et al., 2006).

Fluctuations in fatty acid composition during the light–dark cycles have been described previously in photosynthetic tissues, supporting the results described here for sunflower seeds. Thus, diurnal fluctuations have been described in 18 carbon unsaturated fatty acid contents in the lipids of developing spinach leaves, where the proportion of oleic increases during the day and declines during the night (Browse et al., 1981). It has also been observed that the linoleic and linolenic content in plantlets of cotton increases during the dark period with respect to the content found in the middle of the light period (Rikin et al., 1993). Additionally, the fatty acid profile in *A. thaliana* leaves showed large diurnal variations, with oleic acid increasing twofold during the light period and decreasing during the night (Ekman et al., 2007).

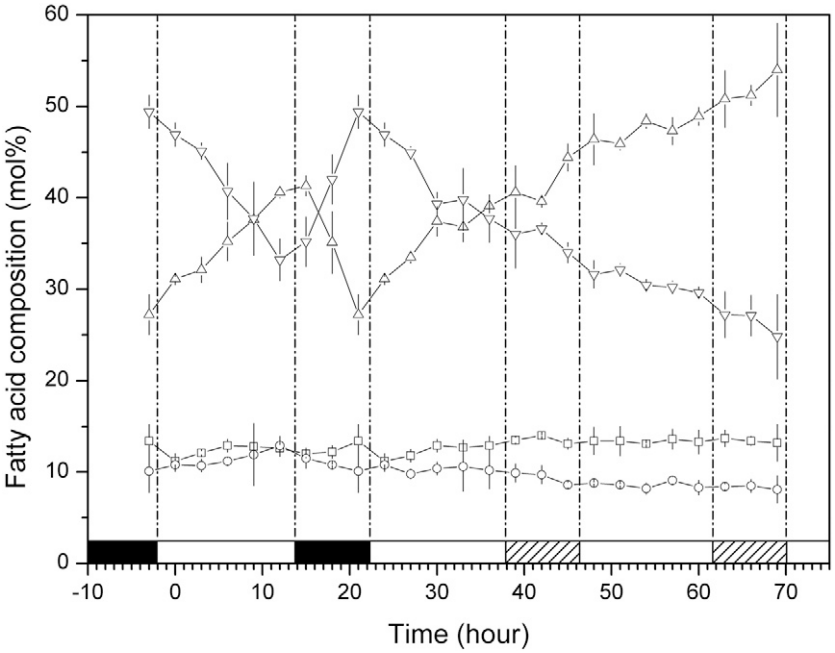

Figure 3. Diurnal variations in the fatty acid composition of total seed lipids. Plants of the CAS-6 sunflower line were grown in culture chambers, 25/15°C, 16-h photoperiod, and 300 μmol m^{-2} s^{-1}, and were switched to conditions of continuous light and temperature (25°C). At least three seeds were sampled every 3 h, and the fatty acid composition was determined in the total oil by gas-liquid chromatography. The results are expressed as the percentage of the mass of three different plants ± SE. Palmitic (○), stearic (□), oleic (△), and linoleic (▽) acids.

CONCLUSIONS

In contrast to photosynthetic tissues, nongreen heterotrophic seeds, such as sunflower seeds, cannot directly sense the presence or absence of light; thus, it is possible

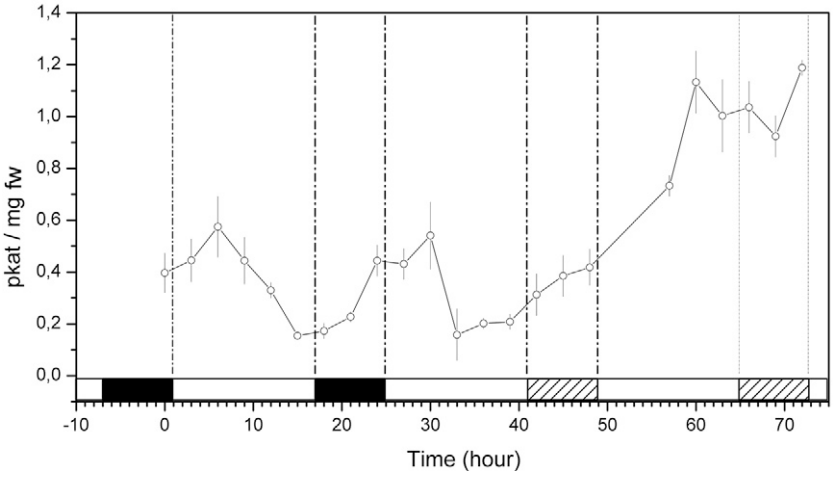

Figure 4. Rhythmic oscillation of the stearoyl-ACP-desaturase (SAD) activity in sunflower seeds. Plants of the CAS-6 line were grown in culture chambers, 25/15°C, 16-h photoperiod, and 200 μmol m^{-2} s^{-1}, and were switched to conditions of continuous light and temperature (25°C). The seeds were sampled every 3 h, and the SAD activity was measured as described in "Materials and Methods." Diurnal period (*x* axis, white bar), nocturnal period (black bar) and subjective night (hatch-marked bar). FW, fresh weight.

that this information reaches them in the form of substrates for the synthesis of reserve substances. Both fatty acids and lipids can be considered among such substrates, and their accumulation depends on photosynthesis or on the nocturnal degradation of the starch accumulated in the leaves during the photosynthetic process. In addition to the possible diurnal–nocturnal effect produced by the presence of these substrates, other regulatory processes have been described. These involve the regulation by transcription factors in seeds, by the partition of the carbon skeleton, activating a complete battery of enzymes. It is possible that the effect observed on SAD activity in conditions of constant light and temperature, and with a virtually continuous supply of substrate, corresponds to this type of regulation. Evidence for noncircadian light–dark-regulated expression of heat shock proteins has been demonstrated in spinach leaves (Li and Guy, 2001), where the expression peak precedes the daily thermoperiod maximum. Likewise, the effects observed on the oleic–linoleic pair may correspond better to the temperature regulation of the corresponding enzymatic activity than to a possible circadian rhythm. The increase in the rate of oleic acid desaturation observed in the middle of the night indicates the influence of night temperature on the desaturation activity in sunflower seeds.

Acknowledgments

Thanks are due to D. S. Cabrera and M. C. Ruiz for their skilful technical assistance. D.R. held a FOMEC scholarship during this study. This work was supported by MEC and FEDER project AGL 2005-00100.

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