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Temperature-dependent endogenous oxygen concentration regulates microsomal oleate desaturase in developing sunflower seeds

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Abstract

Oleoyl-phosphatidylcholine desaturase (FAD2) is a key enzyme involved in fatty acid desaturation in oilseeds, which is affected by environmental temperature. The results of this study show that FAD2 is regulated in vivo via temperature-dependent endogenous oxygen concentrations in developing sunflower (Helianthus annuus L.) seeds. By combining in vivo oxygen profiling, in situ hybridization of FAD2 genes, an assay of energy status, fatty acid analysis, and an in vitro FAD2 enzyme activity assay, it is shown that: (i) the oil-storing embryo is characterized by a very low oxygen level that is developmentally regulated. Oxygen supply is mainly limited by the thin seed coat. (ii) Elevations of external oxygen supply raised the energy status of seed and produced a dramatic increase of the FAD2 enzyme activity as well as the linoleic acid content. (iii) A clear negative correlation exists between temperature and internal oxygen concentration. The changes occurred almost instantly and the effect was fully reversible. The results indicate that the internal oxygen level acts as a key regulator for the activity of the FAD2 enzyme. It is concluded that a major mechanism by which temperature modifies the unsaturation degree of the sunflower oil is through its effect on dissolved oxygen levels in the developing seed.

Key words: Helianthus annuus, linoleic acid, oleic acid, oleoyl-phosphatidylcholine desaturase (FAD2), oxygen regulation, sunflower seeds, temperature regulation.

Introduction

The characteristics of fats and oils are considerably dependent on their fatty acid composition. As the unsaturated fatty acids, oleic, linoleic, and α-linolenic acids, are the major fatty acids in storage triacylglycerols of oilseeds and oil-containing fruits, the degree of unsaturation of vegetable oils determines their relevant technological and nutritional properties (Márquez-Ruiz et al., 1999; Cunnane, 2003).

Fatty acid biosynthesis in oilseeds is localized in the plastids. It is initiated with acetyl-CoA as substrate and is catalysed by the co-ordinated action of two enzymes: acetyl-CoA carboxylase and the dissociable multienzymatic complex fatty acid synthase (Harwood, 2005), yielding mainly palmitoyl-ACP and stearoyl-ACP. Most of the stearoyl-ACP is desaturated by the soluble stearoyl-ACP desaturase to oleoyl-ACP, which is the main product of the plastidial fatty acid biosynthesis. These acyl-ACPs are hydrolysed to free fatty acids, activated to the corresponding acyl-CoAs, and exported to the cytosol, to be incorporated into glycerolipids. The enzymes responsible for the extra-plastidial desaturation of oleic acid into linoleic acid, and then into α-linolenic acid, are oleoyl-phosphatidylcholine desaturase (FAD2) and linoleoyl-phosphatidylcholine desaturase (FAD3), respectively. Both are membrane-bound enzymes located in the endoplasmic reticulum that catalyse reactions involving the concomitant reduction of molecular oxygen to water and require NADH, NADH-cytochrome b5 reductase, and cytochrome b5 as the electron donor system (Shanklin and Cahoon, 1998).

The proportion of these unsaturated fatty acids can be modified by the growth temperature during oilseed
development, yielding oils with a wide and unwanted variation of their degree of unsaturation, which depends on the environmental conditions (Canvin, 1965). Low temperatures increase the polyunsaturated fatty acid content of oilseeds. However, depending on the plant species, the range of this temperature effect is pronounced to varying degrees. In safflower seeds, the linoleic acid content is weakly influenced by environmental temperature (Knowles, 1972), whereas in sunflower (Helianthus annuus L.) seeds it is much more temperature dependent (Harris et al., 1978; Lajara et al., 1990). In the case of soybean and oilseed rape, a moderate temperature effect on the polyunsaturated fatty acid composition has been reported (Trémolières et al., 1982; Wolf et al., 1982). The development of new oilseed varieties with a temperature-independent fatty acid profile is an important biotechnological aim, since the maintenance of an optimal fatty acid composition is critical to retain the value and specific applications of any vegetable oil. To achieve that, it is crucial to understand the mechanisms by which temperature modifies the polyunsaturated fatty acid profile of oilseeds.

In higher plants, different mechanisms have been suggested to explain the temperature regulation of desaturase activity. In addition to transcriptional (Gibson et al., 1994; Kodama et al., 1997; Berberich et al., 1998; Wang et al., 2006) and post-transcriptional mechanisms (Horiguchi et al., 2000; Matsuda et al., 2005), early studies proposed a regulatory role for oxygen, the common substrate of all the desaturases, since the higher solubility of this gas in water at low temperature could increase the total desaturase activity by increasing the availability of oxygen as substrate in non-photosynthetic tissues (Harris and James, 1969). Although varying oxygen concentrations in vitro using microsomes from developing safflower seeds did not affect FAD2 activity (Browse and Slack, 1983), this mechanism might act in vivo in systems where oxygen availability could be limiting for the desaturase, such as cultured sycamore cells (Rebeille et al., 1980) and non-photosynthetic microorganisms such as yeast (Vasconcelles et al., 2001) and amoeba (Thomas et al., 1998; Rutter et al., 2002). Notably, recent studies have shown that developing seeds are characterized by a hypoxic internal environment (Geigenberger, 2003), including some oilseeds such as rape (Vigeolas et al., 2003), and soybean and maize (Rolletschek et al., 2005a, b), where lipid biosynthesis is limited by oxygen availability. These data indicate the critical role of oxygen in the physiology of seed development.

In sunflower seeds, it has been suggested by our group that not only does temperature affect the de novo oleate biosynthesis and its mobilization from pre-formed triacylglycerols, thus modifying the available amount of oleate for desaturation (Garcés et al., 1994; García-Díaz et al., 2002), but it also regulates the FAD2 activity by two different mechanisms (García-Díaz et al., 2002): (i) a long-term direct effect mostly related to the low thermal stability of the enzyme (Martínez-Rivas et al., 2003); and (ii) a short-term indirect effect by which temperature determines the availability of oxygen, which, in turn, regulates the FAD2 activity level. The direct temperature effect was studied further (Sánchez-García et al., 2004), and it was shown that it is caused mainly by the low thermal stability of the FAD2-1 isof orm, which is highly and exclusively expressed in developing seeds, whereas the FAD2-2 and FAD2-3 genes are weakly expressed in all the tissues studied (Martínez-Rivas et al., 2001). The simultaneous action of both effects explained the long well-known difficulty in understanding the definite influence of growth temperature on the oleate to linoleate ratio in sunflower seed oils. Studies using safflower seeds have confirmed the presence of both mechanisms (Esteban et al., 2004).

The main goal of this work was to investigate the proposed regulatory role of oxygen in the indirect temperature effect on the FAD2 activity in developing sunflower seeds. Toward this end, techniques for oxygen profiling across developing seeds using oxygen-sensitive microsensors were combined with spatial expression analysis by in situ hybridization of FAD2 genes. In addition, metabolic assays of energy status and fatty acid composition, and in vitro assays for FAD2 enzyme activity were performed. Collectively, our results show that at physiological temperatures, the sunflower seed tissues are fairly hypoxic and the changes in the internal oxygen level regulate the FAD2 activity and, thus, the degree of unsaturation of the sunflower oil.

**Materials and methods**

**Plant material**

Sunflower (H. annuus L. cv. HA-89) seeds were provided by Dr JM Fernández-Martínez, IAS, CSIC, Córdoba, Spain. Plants were cultivated in a growth chamber with a 16 h photoperiod, photon flux density of 300 µmol m⁻² s⁻¹ (fluorescent lamps; Cool white and Gro-lux; Sylvania) at 25/15 °C (day/night). Capitula, detached achenes, or peeled seeds (achenes without pericarp and seed coat) were used for the experiments. The term seed, although botanically unsuitable for sunflower, is utilized due to its widespread use in the literature on oilseed plants. The outermost two rows of the capitulum opened and anthesed on the same day. Achenes were collected from these two rows at defined days post-anthesis (DPA). For details see the figure legends.

**Determination of endogenous oxygen levels**

The oxygen concentration inside seeds was measured using oxygen-sensitive microsensors (50 µm tips, Presens, Neuburg, Germany) as detailed earlier (Rolletschek et al., 2002). The detached achene was carefully moved into a fixed position. Then, the proximal pericarp tip was cut and the electrode was inserted into the seed using a micromanipulator. In some cases, achenes were incubated in a stream of 60% or pure oxygen. Data were normalized (log-transformed) followed by t test (P <0.05).
Subcellular fractionation

The achenes were peeled by removing the pericarp and the seed coat, and immediately homogenized as follows. Ten peeled seeds (~0.25 g) were ground in a pre-cooled mortar with 10 ml of 50 mM HEPES buffer (pH 7.2) containing 0.6 M sorbitol, 40 mM Na-ascorbate, 1 mM Na₂EDTA, and 1 mM MgSO₄. All manipulations were done at 4 °C. The homogenate was kept at −20 °C or centrifuged at 10 000 g for 5 min. The fat layer was discarded and the supernatant was centrifuged for 1 h at 100 000 g. The pellet containing the microsomal fraction was resuspended in 1 ml of grinding medium and stored at −80 °C.

Lipid extraction and analysis

Aliquots of the homogenate (0.5 ml) were mixed with 1.25 ml of chloroform:methanol:acetic acid (50:50:1, v/v/v) and shaken. Total lipids were recovered from the lower phase, evaporated to dryness with nitrogen, and converted into the corresponding fatty acid methyl esters by heating at 80 °C for 1 h in a 2 ml solution of methanol:toluene:H₂SO₄ (80:20:2, v/v/v) (Garceés and Mancha, 1993). After cooling, the methyl esters were extracted with 2 ml of heptane and analysed by gas-liquid chromatography (GLC) (García-Díaz et al., 2002).

In vitro assay of enzyme activities

The in vitro assay of the FAD2 activity was carried out as described by García-Díaz et al. (2002) using 50 μl of microsomal suspension (corresponding to ~12 mg of fresh seed tissue and 50 μg of protein), and 1.42 nmol [1-¹⁴C]oleoyl-CoA, with 30 min incubation time. To correct the activity values for substrate dilution of [¹³C]oleoyl-phosphatidylcholine, the amount of endogenous oleoyl-phosphatidylcholine in the different microsomal preparations was determined as follows. The total microsomal lipids were quantified by GLC of the corresponding fatty acid methyl esters using heptadecanoic acid as the internal standard. An additional aliquot of the microsomal lipids was separated by thin-layer chromatography (TLC) on silica gel plates developed with chloroform:methanol:water (85:15:10:3.5), and the endogenous oleoyl-phosphatidylcholine quantified as before. Lysophosphatidylcholine acyltransferase and NADH-cyt b₅ reductase activities were determined according to Sperling et al. (1990).

In situ hybridization

Probes for in situ hybridization were labelled with ChromaTide Alexa Fluor 546-14-UTP (Molecular Probes, Eugene, OR, USA). The FAD2 gene-specific fragments (Martínez-Rivas et al., 2001) were amplified by PCR using standard M13 reverse and forward primers. About 1 μg of the PCR products were used as templates to synthesize fluorophore-labelled RNA using T7 RNA polymerase (antisense probes) or SP6 RNA polymerase (sense probes) with the MAXScript in vitro Transcription Kit (Ambion, Austin, TX, USA). The probes were purified using NucAway Spin Columns (Ambion). Peeled sunflower seeds at 17 DPA were fixed in 4% formaldehyde, embedded in paraplast, and sections were prepared as described by Gotor et al. (1997). The hybridization protocol of Cox and Goldberg (1988) was followed. Tissues sections were observed using a Leica TCS SP2 spectral confocal microscope (Leica Microsystems, Germany) and imaged using the 543 nm line of an argon ion laser in single confocal optical sections. Emitted light was collected through a triple dichroic beam-splitter (TD 488/543/633) and detected after spectral separation in the 550–700 nm range on the PMT1 (pseudo-coloured red). Transmitted light imaging of the samples was simultaneously recorded.

Determination of adenine nucleotides

The achenes were frozen in liquid nitrogen and stored at −80 °C until analysed. Later, they were homogenized with a pestle and mortar and extracted with chloroform-methanol (Soga et al., 2002). Adenine nucleotides were measured after derivatization by high-performance liquid chromatography (HPLC) with fluorescence detection (Rolletschek et al., 2005a).

Sectioning and staining procedures

Toluidine staining was performed on paraplast microsections (10 μm) as described in Borisjuk et al. (1995).

Results

The seed coat strengthens hypoxia within developing sunflower seeds

The oxygen concentration inside developing seeds was analysed using oxygen-sensitive microsensors (Rolletschek et al., 2002). When the sensor was inserted into the seed, the oxygen concentration dropped to approximately half-saturation values within the first 500 μm (Fig. 1A; red arrow in Fig. 1B indicates insertion of the sensor). Local variations of the oxygen level within this micropylar region of the seed were related to a high structural heterogeneity (Fig. 1C). Moving the microsensor towards the lipid-storing embryo resulted in a dramatic decrease of oxygen levels. Within the embryo, minimum levels below 1 μM were detected, probably indicating oxygen limitation (hypoxia). In addition, the oxygen concentration showed no further gradients. Unlike the developing seed, the sponge capitulum tissue harbouring the achenes is green and photosynthetically active. Illumination (300 μE) of this tissue raised the oxygen level from 231.8±2.5 μM to 241.0±2.8 μM due to photosynthetic oxygen release. However, this small rise should not elevate the endogenous oxygen concentration within the seed because much larger changes in the external oxygen environment are necessary to affect the endogenous oxygen level (see below).

Bulk seed tissues possess high diffusional impedance. Thus, the seed structures which are responsible for the possibly restricted gas exchange capacity were determined. Removing the pericarp (Fig. 2B, C) did not affect the endogenous oxygen level (Fig. 2A). However, peeling off the seed coat (Knowles, 1978), to which attached lipoidic residues of integuments and endosperm have been observed (shown with the red arrow in Fig. 2B, C), was followed by an immediate increase in the internal oxygen concentration (Fig. 2A). This clearly identifies this thin coat as a major diffusional barrier in sunflower seeds. The appearance of this coat is observed on entering the storage phase.

To analyse developmental effects, the mean oxygen levels inside seeds at distinct stages were measured. Oxygen concentrations were very low in all stages.
analysed here (Fig. 2D). Nevertheless, a clear developmental pattern was observed, with a significant decrease between 10 DAP and 25 DAP, followed by an increase during the last part of the development process.

Low internal oxygen levels restrict the energy status of seeds

To check that oxygen is a limiting factor for sunflower seed respiration, as previously suggested (García-Díaz et al., 2002), two approaches have been followed. First, intact achenes were incubated with elevated oxygen levels, and the effects on the endogenous oxygen concentration were measured (Fig. 3A). Treatment with an approximately 3-fold atmospheric oxygen level did not affect the oxygen status of seeds. Pure oxygen treatment was necessary to elevate internal levels significantly. This strongly implies that the oxygen supplied is instantly used via an elevated respiratory activity of seeds.

Low internal oxygen levels limit FAD2 activity in sunflower seeds

The removal of the pericarp and the seed coat produces a strong and rapid increase in the FAD2 activity in sunflower seeds, which was attributed to increased oxygen availability (García-Díaz et al., 2002). To confirm this assumption further, sunflower achenes remaining in the capitulum as well as detached achenes were incubated in atmospheres of 100% oxygen for 1, 6, and 24 h. After incubation, the \textit{in vitro} FAD2 activity was determined in the corresponding microsomal fractions and corrected for substrate dilution, since incubation under oxygen for 1, 6, and 24 h brought about a decrease of the endogenous oleoyl-phosphatidylcholine in the microsomes from achenes either remaining in the capitulum (6.4, 7.8, and 6.0 nmol, respectively, Fig. 2D).
respectively) or detached (6.4, 4.6, and 3.2 nmol, respectively), as compared with that at zero time (12.8 nmol). The enzyme activity in sunflower achenes either remaining in the capitulum or detached increased significantly after 1 h incubation (Fig. 4A). This activity level continued increasing for longer times in the case of achenes remaining in the capitulum, but it was already maximal after 1 h for detached achenes. A parallel but delayed effect on the linoleate content of total seed lipids was observed in the same experiment. After 6 h and 24 h incubation, a very strong increase in the amount of linoleic acid was detected (Fig. 4B), indicating the 

in vivo

physiological relevance of the increase in FAD2 activity. However, neither the FAD2 activity nor the linoleate content was modified when the achenes were incubated in 60% oxygen (results not shown), which is in agreement with the fact that this external oxygen concentration did not elevate the endogenous oxygen concentration within the seed (Fig. 3A).

Microsomes isolated from sunflower achenes (zero time) have also been assayed for FAD2 activity, but using in vitro a very low oxygen concentration (1 μM) similar to that found in vivo in sunflower achenes (Fig. 2), instead of saturating oxygen for comparison. The FAD2 activity value obtained in this way was 0.4 nmol linoleic acid (g FW)^{-1} h^{-1}.

Because the reaction for the FAD2 activity assay requires the prior transfer of radiolabelled oleate from oleoyl-CoA to phosphatidylcholine, and is dependent upon cyt b_{5} and its reductase, the possible effect of the internal oxygen level on these enzyme activities was studied. Neither the lysophosphatidylcholine acyltransferase nor the NADH-cyt b_{5} reductase activities showed any change when the seeds were incubated under different external oxygen concentrations (results not shown). Therefore, low endogenous oxygen levels specifically limit FAD2 activity in the seeds. This result is in agreement with that of Rutter et al. (2002) obtained in amoeba.

Spatial distribution of FAD2 transcripts is not related to the oxygen profile inside the sunflower seed

In situ hybridization techniques were used to check if the spatial distribution of the sunflower FAD2 transcripts corresponds to the oxygen map within the seed. FAD2-1 transcript appeared in all the cell types, although a stronger signal was observed in the procambial ring and the radicle tip (Fig. 5A, B). In addition, the FAD2-1 mRNA was
preferentially localized in the palisade parenchyma cells compared with the spongy parenchyma (Fig. 5C–F). Therefore, the expression pattern of the \( FAD2-1 \) gene was not related to the oxygen distribution within the seed (Fig. 1).

On the other hand, \( FAD2-2 \) and \( FAD2-3 \) mRNAs (Fig. 5G, H, and I, J, respectively) had a tissue distribution which was quite homogeneous throughout the seed. The low contrast between the antisense and the background signals is probably due to the previously reported very low expression level of these genes in sunflower seeds (Martínez-Rivas et al., 2001).

**Temperature changes affect internal oxygen levels in the seeds**

The effect of changing temperature on endogenous oxygen levels was tested. Whole sunflower capitula were put into a climate chamber at 10 °C, achenes were removed, and the microsensor was inserted into the seed. The chamber temperature was allowed to rise at 3 °C min\(^{-1}\) and the changes in the seed temperature and internal oxygen level were monitored. As shown in Fig. 6A, the temperature rise clearly reduced the endogenous oxygen concentration, the response being very rapid. At temperatures over 40 °C, the mean oxygen level fell below 0.2 \( \mu \text{M} \). When decreasing the temperature within the growth chamber, internal oxygen levels reached initial values (data not shown), indicating the reversibility of this process. The embryo oxygen concentration can be described as a slightly non-linear function of temperature (Fig. 6B). The temperature rise was accompanied by a steady decrease in the energy status (given by the ATP/ADP ratio) of seeds (Fig. 6C). However, at temperatures above 25 °C the ATP/ADP ratio remained constant. It is concluded that temperature modifications from 10 °C to 40 °C, representing
physiological conditions in the field, cause instant changes in the internal oxygen concentration of seeds.

**Discussion**

**Structural and developmental cues of internal oxygen depletion in sunflower seeds**

This work highlights the endogenous oxygen depletion within developing sunflower seeds (Figs 1,2). The oil-storing embryo is characterized by very low internal oxygen concentrations. Hypoxic conditions (<2 \( \mu \text{M} \)) prevail throughout the entire embryo. Such low levels clearly indicate internal hypoxia (Geigenberger, 2003; Rolletschek and Borisjuk, 2005). Similar low oxygen levels were found in seeds of all monocot and dicot species analysed so far (faba and pea, Rolletschek et al., 2002, 2003; barley, Rolletschek et al., 2004; rape, Vigeolas et al., 2003; wheat, Van Dongen et al., 2004; soybean, Rolletschek et al., 2005b; maize, Rolletschek et al., 2005a). It is noteworthy that the sunflower seed completely lacks any photosynthetic activity. This might cause a higher susceptibility to oxygen deprivation and internal hypoxia as compared with green seeds.

Oxygen supply to the developing embryo depends entirely on diffusion from the ambient air, so is governed by diffusive resistance of seed tissues. Barriers to gas exchange may be the pericarp and testa (barley, Nutbeam and Duffus, 1978; Freeman and Palmer, 1984) as well as cuticula and seed coat (pea, Wager, 1974). In sunflower seeds, the oxygen concentration profiles (Fig. 1A) and the step by step peeling of peripheral tissue layers (Fig. 2A) demonstrated that oxygen uptake is mainly limited by the seed coat, but not by the pericarp. This finding was quite unexpected because the much thicker, multiple layers of the pericarp were assumed to represent the major diffusional barriers. Although the structural and compositional characteristics of the seed coat are unknown, its
lipidous character might be a major cause of this barrier function. It further suggests that future attempts to increase seed oxygen supply should be focused on the biophysical characteristics of this coat.

The mean oxygen concentration found in the oil-storing embryo depended on the developmental stage of the sunflower seed. Similar data come from studies with legume and cereal seeds (Rolletschek et al., 2003, 2005b). Changes in the steady-state oxygen levels found here might be related to two aspects: (i) oxygen delivery, depending on the diffusive resistance of seed tissues; and (ii) endogenous oxygen demand, mainly caused by mitochondrial respiration but also fatty acid desaturation and other processes. Both aspects certainly underlie significant developmental changes. In barley seeds, it was demonstrated that both the mean oxygen concentration and the energy state decline at the onset of the main starch storage phase (Rolletschek et al., 2004). In sunflower seeds, a significant decrease in oxygen concentrations was found in the period between 10 DPA and 25 DPA, in which maximal oil accumulation was reported (Luthra et al., 1991). It is reasonable to assume that respiratory oxygen consumption is likewise increased during the time of high energy-demanding oil biosynthesis.

Oxygen supply restricts respiration and energy status in developing seeds

A marked influence was demonstrated for normal degrees of endogenous oxygen depletion, by experimentally altering oxygen supplies to intact seeds (Fig. 3A). The oxygen demand of developing seeds is not saturated at an ambient (atmospheric) oxygen level. Even 3-fold increases in the exogenous supply did not increase the internal oxygen levels. However, a significant increase in the ATP/ADP ratio was evident under this treatment (Fig. 3B). This clearly indicates a higher respiratory activity in response to higher oxygen supply. More marked elevations of external oxygen concentration eventually raised the embryo oxygen levels above those observed in vivo. Again, this was coupled with significant elevations in the ATP/ADP ratio. Thus, an increasing oxygen supply is apparently balanced by increasing oxygen consumption. Consequently, respiration inside developing seeds is considered to be non-oxygen-saturated under in vivo conditions, i.e. it is oxygen-limited. Enhanced oxygen availability is coupled with higher ATP levels, increased ATP/ADP ratio, and thus elevated overall energy status of seed tissue.

As well as such effects on the respiratory activity, oxygen is known to play a pivotal role in assimilate partitioning and storage metabolism. For example, the analysis of diverse dicot seeds showed that endogenous oxygen limitation affected resource allocation among storage products (Borisjuk et al., 2003; Rolletschek et al., 2003). In addition, mainly lipid but not starch storage metabolism is limited by low internal oxygen levels, as demonstrated for oilseed rape embryos (Vigeolas et al., 2003) and maize kernels (Rolletschek et al., 2005a). In this work it is further demonstrated that lipid unsaturation is also affected by endogenous oxygen in oilseeds.

Fig. 6. Temperature effects on internal oxygen concentration and energy status of sunflower seeds. (A) Simultaneous monitoring of changes in temperature and internal oxygen levels measured in an embryo. (B) Regression plot for the data given in (A). (C) Changes of the ATP/ADP ratio in response to temperature. Data are given as means \( \pm SD (n=5) \). A non-linear regression line is fitted to the data.
FAD2 activity is regulated by oxygen availability within the sunflower seed

Previous studies suggested a role for oxygen in the regulation of the sunflower FAD2 enzyme (García-Díaz et al., 2002; Martínez-Rivas et al., 2003). In the present work, it has been demonstrated that elevating the endogenous oxygen levels in sunflower seeds produced a strong increase in FAD2 activity (Fig. 4A). The low activity level detected in vitro in microsomes isolated from sunflower achenes (zero time) is not caused by the in vivo reduced availability of oxygen acting merely as a substrate for the enzyme. If this were the case, when the activity was measured in vitro with saturating oxygen concentration, a high activity level similar to that for achenes incubated with pure oxygen should be observed. Therefore, oxygen is not only acting as a substrate reducing FAD2 activity due to its limitation in vivo, but is also involved in a specific mechanism that regulates the enzyme activity level. To estimate the relative importance of both effects, the FAD2 activity levels in microsomes from sunflower achenes (zero time) assayed in vitro with a very low oxygen concentration (1 μM) similar to that found in vivo in sunflower achenes (Fig. 2), and with saturating oxygen [0.45 nmol and 5 nmol linoleic acid (g FW)^−1 h^−1, respectively] were compared with those from sunflower achenes remaining in the capitulum incubated for 6 h with 100% oxygen [63 nmol linoleic acid (g FW)^−1 h^−1, Fig. 4A]. These data show that over and above the 12-fold increase of the FAD2 activity due to the use of saturated oxygen as substrate, the occurrence of this specific mechanism causes an additional 12-fold increase, indicating its high relevance.

The higher FAD2 activity detected in detached achenes, as compared with that of attached achenes, could be due to the higher external surface that is in contact with the external 100% oxygen atmosphere. The increase in FAD2 activity was accompanied by an increase in the linoleic acid content (Fig. 4B), confirming that this regulation notably modifies the degree of unsaturation of the sunflower oil.

Not only the FAD2 enzyme, but also other enzymes involved in oilseed lipid biosynthesis seem to be regulated by oxygen availability. Recently, it has been shown in oilseed rape that diacylglycerol:acyltransferase activity increased at elevated external oxygen and correlated with increased triacylglycerol biosynthesis (Vigeolas et al., 2003).

Interestingly, during seed development, the period of decreasing endogenous oxygen level (10–25 DPA) (Fig. 2D) coincides with that of the highest expression of the seed-specific FAD2-1 gene (Martínez-Rivas et al., 2001) and maximal FAD2 enzyme activity (Garcés and Mancha, 1991), as well as that of other desaturases that also use oxygen as substrate, such as the stearoyl-ACP desaturase (Salas et al., 2004). Therefore, a competition between respiration and desaturation for the low amount of oxygen present in the embryo seems to occur. Thus, the present mechanism could act by maintaining FAD2 activity at low levels to prevent anoxia, since oxygen availability is a key factor for seed development (Roll-etschek et al., 2002; Geigenberger, 2003). Regarding the molecular mechanism, no correlation was found between the spatial distribution of FAD2-1 transcripts (Fig. 5) and the oxygen concentration profile inside the sunflower seed (Fig. 1), suggesting that the transcription of the gene is not regulated by oxygen. However, further work needs to be done to elucidate the mechanism completely. On the other hand, the high expression level of the FAD2-1 gene in the proximal end of the seed (Fig. 5A) could explain the previously reported gradient of the oleic to linoleic acid ratio along the sunflower seed (Fernández-Moya et al., 2003).

Temperature affects internal oxygen levels, energy status, and the FAD2 activity level of sunflower seeds

Two different mechanisms have been proposed by which temperature regulates the FAD2 activity in sunflower seeds: a direct and an indirect effect (García-Díaz et al., 2002; Martínez-Rivas et al., 2003). In support of the second mechanism, a clear negative correlation between temperature and internal oxygen concentration has been shown in this work (Fig. 6B). The changes occurred almost instantly as the temperature increased (Fig. 6A) and the effect was fully reversible when the temperature was decreased again. A parallel negative correlation was also observed between temperature and FAD2 activity level (Martínez-Rivas et al., 2003). In the present study, it is also shown that under physiological temperature conditions the seed tissues are fairly hypoxic, with oxygen concentrations as low as 0.1–0.2 μM at 40 °C. At 30 °C and 10 °C, the oxygen concentrations were approximately 0.5 μM and 2 μM, respectively. As previously reported, a strong olate desaturation occurred in detached sunflower achenes incubated for 24 h at 10 °C, while no desaturation occurred at 30 °C (García-Díaz et al., 2002). In addition, temperature changes from 10 °C to 30 °C or vice versa brought about a fast and dramatic decrease or increase in the level of FAD2 activity, respectively (Martínez-Rivas et al., 2003). Therefore, it could be suggested that the internal oxygen level acts as a switching system to exchange high and low activity levels of the FAD2 enzyme, the critical value being at a point around 1 μM. This mechanism is of biotechnological interest as it seems to be the main factor responsible for the final contents of oleic and linoleic acids in sunflower seed oils. It is also of outstanding physiological significance since it acts under field growth conditions.

As far as other oilseeds are concerned, both the direct and the indirect mechanisms were also shown to occur in
safflower achenes submitted to changes in temperature or external oxygen concentration (Esteban et al., 2004). However, unlike sunflower, the removal of the pericarp and seed coat did not increase the FAD2 activity, which remained at the same high levels. This highlighted the physiological relevance of the presence of a diffusional barrier capable of modulating the internal oxygen concentration. Interestingly, green oilseeds, that exhibit a much higher endogenous oxygen level than sunflower, such as soybean (Rolletschek et al., 2005b) or rape (Vigeolas et al., 2003), are characterized by a moderate temperature effect on the degree of unsaturation of the corresponding oils.

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References


Temperature-dependent oxygen regulation of sunflower FAD2


