High Oleic Sunflower: Studies on Composition and Desaturation of Acyl Groups in Different Lipids and Organs

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Z. Naturforsch. **45c,** 166–172 (1990); received November 20, 1989

Triacylglycerols, Galactolipids, Phosphatidylcholine, Cotyledons, Leaves, Roots

A selection of lipids from achenes, cotyledons after germination, roots and leaves of normal and high oleic varieties of sunflower were analyzed with regard to their fatty acid profiles. The lipids included triacylglycerol and phosphatidylcholine as ER-made components and mono- and digalactosyl diacylglycerol as plastid-localized glycolipids. A comparison of fatty acid patterns showed that the block in oleate desaturation of the high oleic variety is confined to the ER of fat accumulating embryos, but that upon germination the oleate desaturation in the cotyledonary ER is rapidly derepressed. These data are supported by enzymatic experiments. In microsomes from maturing fruits of the high oleic variety oleoyl-phosphatidylcholine desaturase could not be detected, whereas oleoyl-CoA : lyso-phosphatidylcholine acyltransferase and components of the microsomal electron transport chains were not affected. A correlation in the expression of desaturation blocks in seed and root fatty acids as observed in mutants of other species was not observed which, therefore, cannot be generalized. Our data are discussed in terms of the existence of two ER-specific oleate desaturase activities.

Introduction

Plants direct different fatty acids with unique functional groups at various positions along the carbon chains [1, 2] into TAG accumulating in reserve organs. Many of these fatty acids are suitable for chemical derivatization, and ideal oleochemicals for large scale processing would be homogeneous TAG with only one type of acyl constituent [3]. But in most cases, the fatty acids of interest are mixed with other ones which would interfere with a specific derivatization. Therefore, several attempts have been made to select oil crops having only a single type of esterified fatty acid in reserve lipids. As a result of such screening, high oleic varieties have been established in sunflower [4] and peanut [5]. 80–90% of their TAG fatty acids are accounted for by 18:1, which in these varieties is increased at the expense of 18:2. The high oleic varieties are not designed for human consumption for which the normal sunflower or peanut oils with a mixture of 16:0, 18:1 and 18:2 are perfectly suited.

From a biochemical point of view, these high oleic varieties are very interesting, because they offer the possibility to study some details of an ER-located 18:1 desaturase activity which is apparently missing in these fruits. On the other hand, a lack of 18:1 desaturation in the ER could be caused by a defect in the desaturase itself or in ancillary enzymes cooperating with the desaturase [6]. A detailed analysis of different, compartment-specific lipids from fruits, leaves and roots should show, whether the block in 18:1 desaturation is confined to a particular compartment in a specific tissue. These investigations are related to questions regarding the existence of different desaturase genes and/or their control by independent regulatory units. In the following we report lipid analyses and enzymatic studies carried out with normal and high oleic sunflower varieties which may contribute to an answer of the above mentioned questions.

Abbreviations: DGD, digalactosyl diacylglycerol; MGD, monogalactosyl diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerol; 16:0, 18:0, 18:1, 18:2, 18:3, palmitic, stearic, oleic, linoleic, linolenic acid.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0300–0166 $ 01.30/0

Experimental Part

Plants

The plant material was derived from sexual crosses between selections of the open-pollinated

variety "Pervenets" and the inbred line HA 89 [7]. Progenies of these crosses were propagated by self-pollination over three successive generations (F_1, F_2, F_3) and selected for high-oleic content, *i.e.* 88% or more. An inbred progeny, the self-fertile line sf23/6, was planted in the experimental field at Groß-Gerau (near Frankfurt/Main) in 1989; sowing date was April 5th, 1989 and seedlings emerged on April 10th. For comparison, a low-oleic (high-linoleic) sunflower hybrid, "Frankasol", was grown adjacent to the high-oleic material, *i.e.* at the same time and in the same place. Average monthly temperatures during the vegetation period were as follows (minima–maxima in parentheses): May 16.4 °C (8.4–23.2), June 17.4 °C (10.5–23.0), July 19.9 °C (13.8–25.8), August 18.5 °C (12.9–24.6). It is well known that the temperature during TAG accumulation has a strong effect on the 18:1/18:2 ratio [8]. For analyses of developing sunflower fruits, complete immature heads were collected on August 1st, 1989, when flowering of the entire heads had been completed, *i.e.* 114 days after emergency.

Other plants of line sf23/6 and *cv.* "Frankasol" were maintained in the field until their full maturity. Then achenes were harvested, dried, cleaned and used for further analyses.

Cotyledons, roots and leaves

Fruits were planted in vermiculite and germinated in a phytotron under short day conditions. Cotyledons (after removal of hypocotyls and roots) were harvested after 2 (very young), 6 (young) and 10 days (green) of germination. After two days of germination the cotyledons were still mostly enclosed by the pericarp and contained, if at all, only a trace of chlorophyll. Roots were obtained from young plants grown for 8 weeks. The first leaves were harvested after growth of seedlings for 18 days.

Lipid extraction and analysis

Achenes (10 g) were first kept in boiling water for 7 min and then freed from hulls [9]. The remaining material (mainly embryos) was homogenized in a mortar with the aid of sand in 50 ml of isopropanol. After addition of $CHCl_3$ (100 ml) the mixture was kept overnight. Then 38 ml of NaCl solution (0.45% in water) was added and the mixture shaken. After phase separation the subphase was evaporated to dryness in a rotary evaporator and subsequently partitioned between 75 ml of petroleum ether and 75 ml of MeOH (95% in water). After removal of the upper phase, the subphase was reextracted with 50 ml of petroleum ether. The combined petroleum ether fractions were evaporated to dryness and used for separation of TAG by TLC in petroleum ether/diethyl ether/acetic acid 70/30/0.5 on precoated silica gel 60 plates. Lipids on TLC plates were detected under UV after spraying with anilinonaphthalene sulfonate (0.2% in MeOH).

The methanolic subphase was evaporated to dryness in a rotary evaporator, dissolved in a small volume of $CHCl_3$/MeOH 2/1 and used for separation of polar lipids by TLC in $CHCl_3$/MeOH/acetic acid/water 85/15/10/3.5. Lipids recovered after the first run by phase partitioning were rechromatographed in $CHCl_3$/MeOH 85/15 (MGD) or $CHCl_3$/MeOH/acetic acid 65/25/8 (DGD and phospholipids). Fatty acid *p*-bromophenacyl esters were prepared and analyzed by reverse-phase HPLC as described before [10].

Leaves, roots and cotyledons (after different times of germination) were first kept in boiling water for 2 min and then homogenized in 6 ml of $CHCl_3$/MeOH 2/1 using an Ultraturrax. After phase partitioning with 1.5 ml of NaCl solution the subphase was processed as described above.

Preparation of microsomes

Cotyledons from developing sunflower fruits (10 g) of both varieties grown for 16 weeks in the same field were ground in a mortar in 5 ml of ice-cold grinding medium [11] (0.33 M sucrose, 0.1% (w/v) bovine serum albumin, 2000 units catalase/ml, 0.1 M phosphate buffer pH 7.2). The brei was diluted with 35 ml of additional grinding medium and filtered through four layers of miracloth. The filtrate was centrifuged for 20 min at 18,000 × *g*, the resultant pellet discarded and the supernatant fraction layered on a cushion of glycerol (0.5 ml) in a 38 ml-centrifuge tube. After centrifugation for 1 h at 100,000 × *g* in a swing-out rotor the supernatant solution was removed by aspiration and the microsomal band on top of the glycerol cushion recovered together with the glycerol for storage at

−20 °C. The yield of microsomal protein [12] was about 4 mg per 10 g of fruits.

Enzymatic assays

NADH- and NADPH-cytochrome c-reductase were assayed [13] using 0.5−15 µg of microsomal protein in a total volume of 0.8 ml containing 0.1 M phosphate buffer pH 7.2, 1.2 mM KCN and 125 µM cytochrome *c*. The reactions were started by the addition of 1 mM NADH or 1.25 mM NADPH. Rates were calculated from the reduction of cytochrome *c* recorded at 550 nm using an extinction coefficient [14] of 18.5 mM^{-1} cm^{-1}. Mitochondrial contamination, checked by addition of 50 µM rotenone or antimycine dissolved in ethanol, was negligible.

NADH-ferricyanide reductase was measured in the same system as described above replacing cytochrome *c* by 1 mM $K_3Fe(CN)_6$. Rates were calculated from the reduction of ferricyanide recorded at 420 nm and using an extinction coefficient [15] of 1.02 mM^{-1} cm^{-1}. Protein linearity was observed up to 2.5 µg of microsomal protein per assay.

Oleoyl-CoA:lyso-PC acyltransferase was assayed [11] in a final volume of 250 µl containing 0.1 M phosphate buffer pH 7.2, 0.25 mM CoA (as lithium salt), 8 mM ATP, 2000 units catalase, 100 µg lyso-PC, 1% (w/v) bovine serum albumin and 23.7 µM oleoyl-CoA including 2.0 kBq of [1-^{14}C]oleoyl-CoA. Reactions were started by the addition of microsomal protein (25−100 µg in glycerol). After 30 min the reaction was stopped by addition of $CHCl_3$/MeOH 1/1 (2.5 ml) and water (1 ml). After mixing and phase separation the subphase was withdrawn and blown to dryness in a stream of argon. The lipids were redissolved in a small volume of $CHCl_3$/MeOH 1/1 and separated by TLC in $CHCl_3$/MeOH/25% NH_4OH 65/35/5. Lipids were located as described above and PC spots scraped off into vials for subsequent scintillation counting.

Oleoyl-PC desaturase was measured after first incubating microsomes with oleoyl-CoA in the above described system to label PC followed by NADH-induced desaturation of PC-linked oleoyl groups [11]. Microsomes (25−100 µg of protein) were incubated exactly as described above for 30 min. Then 3 mM NADH was added and incubation continued for further 30 min. The reactions were stopped as described above, PC separated by TLC and subjected to transmethylation in MeOH/H_2SO_4 96/4 (v/v) for preparation of fatty acid methyl esters which were separated by radio-HPLC as described before [16]. Calculation of rates did not take into account any dilution of labelled oleoyl groups by unlabelled, PC-linked oleoyl residues and, therefore, may underestimate the actual activities.

Results and Discussion

The analysis of membrane lipids from fat-storing reserve organs requires the removal of TAG which account for about 98% of the lipids in extracts of such tissues [8]. We separated these apolar compounds by a simple phase partition between petroleum ether and 95% methanol. The polar lipids were recovered quantitatively in the methanolic phase and subsequently separated by TLC and rechromatographed in different solvents. Although we did not carry out a quantitative analysis, we noticed that the polar lipid fraction from fruits contained more DGD than MGD reverting the ratio known from green tissues and indicating that galactolipids from these organs originate mainly from envelopes of plastids lacking thylakoids [17].

In addition to fruits we also analyzed cotyledons after different times of germination to see, whether this organ can regain desaturase activity not present during lipid accumulation. It would indicate repression and derepression of this activity within the same cotyledonary cell, if one assumes that cell number in sunflower cotyledons does not change during germination as demonstrated for other seeds during epigeous germination [18, 19]. For similar reasons leaf and root lipids were analyzed. A comparison of lipid desaturation in these organs should not only deal with organ specific expression of desaturase activities in the case of sunflower and its high oleic mutant, but it should also show, to what extent data from similar experiments with mutants from other species can be generalized. Mutations affecting desaturases in *Arabidopsis* chloroplasts have been found to be expressed only in leaves and not in roots and seeds [20, 21], although the minor plastid lipids of these tissues have not been analyzed. On the other hand, the block of 18:2 desaturation found in seed TAG of a

flax mutant did not show up in leaf lipids from chloroplasts and extraplastidic membranes [22]. Furthermore, a reduction of 18:2 desaturation in soybean TAG was claimed to be also expressed in roots but not in leaves [23], and recently a similar situation has been found in *Arabidopsis*, where a block of 18:2 desaturation in seeds was also found in roots [24]. To cover such subcellular desaturation alternatives and possible tissue correlations we analyzed PC and galactolipids from fruits, roots and leaves of normal and mutant sunflower varieties.

The level of unsaturated fatty acids present in ER- (PC and TAG) and plastid-made lipids (MGD, DGD) from the fruits of the two varieties shows minor, but significant differences (Table I). In the normal variety, we noticed nearly identical patterns in PC and TAG in agreement with the involvement of PC in the biosynthesis of TAG [25]. As major fatty acids both lipids contain 73% of 18:2, whereas 18:3 is missing. In the plastid lipids MGD and DGD the level of 18:1 is decreased (3–9%) in favour of a slightly increased level of 18:2 (80–84%) and a small but characteristic proportion of 18:3 (4–10%). In view of recently developed schemes for the cooperation of subcellular compartments in lipid biosynthesis [26] we interpret these data as showing that plastids in sunflower fruits receive a C_{18}-fatty acid mixture similar to that found in PC, but due to the operation of separate 18:1- and 18:2-desaturase activities in plastids [16, 27] the imported mixture is metabolized to a slightly higher degree of unsaturation. The desaturases in the plastids of fruits are not very active, but their capacity is sufficient to produce some 18:3 which is absent from PC and TAG. Therefore, the different desaturase sets in plastids and ER of fat accumulating cotyledons of sunflower are not under common control.

Upon germination of achenes of the normal variety, plastid-localized desaturases become highly active in cotyledons, apparently due to the differentiation of chloroplasts and thylakoids [17]. There is a large shift towards higher unsaturation of fatty acids in galactolipids after two days of germination increasing the level of 18:3 to 76–81%. Even in PC (but not in TAG) a small proportion of 18:3 (5%) is found. These germination-induced changes were followed through two additional stages of cotyledon development up to full greening, but only those found in the first stage after two days of germination are shown in Table I, since the other two did not deviate significantly, but supported and accentuated the rapid activation of desaturation. At this point it may be mentioned that the enzymatic equipment for conversion of fats into carbohydrates is fully expressed after two days of germination in sunflower cotyledons [28].

Table I. Composition of fatty acids (mol %) in four different lipids from fruits (fr), very young cotyledons after two days of germination (ct), leaves (lv) and roots (rt) of high oleic (high) and normal (normal) varieties of sunflower.

Lipid	Organ	Variety	Fatty acids 16:0	18:0	18:1	18:2	18:3
TAG	fr	high	6	4	86	4	–
		normal	6	5	16	73	–
	ct	high	2	7	89	2	–
		normal	7	5	20	68	–
PC	fr	high	3	1	92	4	–
		normal	10	3	14	73	–
	ct	high	5	3	26	61	5
		normal	17	6	5	67	5
	lv	high	22	3	8	47	20
		normal	27	2	4	42	25
	rt	high	20	2	2	56	20
		normal	24	1	3	59	13
MGD	fr	high	1	1	83	13	2
		normal	5	3	9	80	3
	ct	high	2	1	33	17	47
		normal	3	2	2	12	81
	lv	high	1	–	1	5	93
		normal	1	–	1	7	91
	rt	high	6	1	3	44	46
		normal	9	2	5	47	37
DGD	fr	high	2	1	62	27	8
		normal	2	1	3	84	10
	ct	high	1	2	52	7	38
		normal	2	3	2	17	76
	lv	high	9	1	2	5	83
		normal	14	2	2	6	76
	rt	high	27	6	7	48	12
		normal	24	2	3	55	16

In comparison to these data from the normal plants, the fruits of the high oleic variety showed some deviations, but also some interesting similar-

ities. As expected TAG and PC have inverted ratios of 18:1/18:2 with 18:1 dominating and ranging from 85–92%, whereas 18:2 is reduced to 3–4%. This indicates a severe, but not complete block of 18:1 desaturation in the ER of this variety. The plastids which receive originally PC-linked fatty acids for glycolipid biosynthesis [26], have galactolipids with similarly inverted 18:1/18:2 ratios, but the proportion of 18:2 is higher than in PC and amounts to 27% in DGD. But even more interesting, the proportion of 18:3 is hardly reduced in the galactolipids of the high oleic variety (2–8%) as compared to the normal plants (3–10%). We interpret these data as indicating that the desaturases of plastids in contrast to those of the ER are not affected in the fruits of the high oleic variety.

The analysis of cotyledonary lipids during germination of high oleic fruits reveals a similar shift towards higher unsaturation as found in normal fruits. After two days of germination the galactolipids contain 38–46% of 18:3 and a concomitant decrease of 18:1. These changes are slower than in normal plants, but continue during greening of cotyledons (data not shown) and approach the situation in normal cotyledons after 10 days. Also in PC a dramatic shift in the 18:1/18:2 ratio is observed, since after two days of germination 61% of 18:2 are found and only 26% of 18:1 are left. Further germination time does not change this pattern and demonstrates that upon germination of fruits from normal and high oleic varieties both ER- and plastid-localized desaturases are rapidly derepressed in cotyledons.

This conclusion was supported by an analysis of PE which is absent from chloroplasts [29]. It displayed the same fatty acid composition as PC in fruits and showed the same shift towards increased unsaturation during the three stages of germination (data not shown, but practically identical with the corresponding PC in Table I).

As mentioned above, the changes of desaturase activities in the ER may occur in preexisting and thus persisting cells. Our data are not sufficient to indicate whether one or two desaturase genes for ER desaturation are involved in this change. According to a simple model a stage-specific desaturase gene for 18:1 desaturation in the ER, which is limited in its expression to the phase of TAG accumulation during seed maturation, is affected in the high oleic variety. The 18:1 desaturation immediately observed upon germination could be due to the expression of a different gene for the normal house-keeping desaturation in the ER. This activity is stage-specifically repressed during seed maturation and in normal plants functionally replaced by the fruit-specific desaturase.

The existence of two genes for 18:1 desaturation in the ER may be supported by a comparative analysis of PC from fruits, leaves and roots of the high oleic variety (Table I). PC from roots and leaves contained high proportions of 18:2 and 18:3, which in addition are similar to those from normal plants. This unsaturation would be ascribed to the expression of the normal house-keeping 18:1 desaturase of the ER, the gene of which would not be altered in the high oleic variety. In line with this picture are the data for the galactolipids from these organs which do not show differences between normal and high oleic varieties, since plastid desaturases may be expressed independently from ER counterparts as is also evident from the above mentioned *Arabidopsis* mutants [20, 21]. The high oleic variety of sunflower would thus be comparable to the 18:3-free mutant of flax which also has normal, *i.e.* high levels of 18:3 in leaf lipids [22]. On the other hand, the sunflower mutant has normal desaturation in root lipids and differs in this respect from seed 18:3 mutants of soybean [23] and *Arabidopsis* [24], which express this reduction in desaturation also in roots. These examples may question the existence of general correlations in the expression of desaturases in different organs of different plants.

To conclude this chapter on fatty acid profiles of different lipids from sunflower we would like to mention that some of the components from fruits and roots listed in Table I have occasionally been analyzed before [30, 31]. The reported data compare well with ours with the exception of one variety grown in India which showed significantly different fatty acid patterns [32].

As mentioned at the beginning, a lack of 18:1 desaturation may be due to defects in different enzymes. Desaturation requires incorporation of 18:1 into PC which is the actual substrate of the desaturase [11, 25], and reducing equivalents which are provided by the components of a short electron transport system [6]. To find out which of these enzymes is actually affected in the high oleic variety we measured the relevant activities in

Table II. Comparison of enzymatic activities related to desaturation of oleoyl groups in microsomal membrane preparations obtained from developing achenes of two sunflower varieties. Activities are based on nmol electron acceptor reduced or product formed using different quantities of protein which have been shown to be proportional to reaction rates. The experiments were carried out with two independent preparations of microsomal membranes and data represent means of these two series.

Enzyme	Activity in nmol × min^{-1} × mg $protein^{-1}$	
	Frankasol	High oleic
NADH-cytochrome *c*-oxidoreductase	3100 ± 350	1400 ± 250
NADPH-cytochrome *c*-oxidoreductase	180 ± 60	140 ± 40
NADH-ferricyanide-oxidoreductase	32000 ± 1800	33000 ± 1900
Oleoyl-CoA : lyso-PC acyltransferase	1.88 ± 0.84	1.82 ± 0.62
Oleoyl-PC desaturase	0.102 ± 0.027	not detected

microsomal membranes prepared from maturing fruits of normal and mutant plants (Table II). A comparison of the different activities shows that the desaturase is rate-limiting in fruits of normal plants and undetectable in mutant fruits. The differences in the other activities between normal and mutant fruits are insignificant in view of the absence of the rate-limiting desaturase in the mutants. In spite of the fact, that the mutant variety is not directly derived from the normal Frankasol variety, the activities of the two flavoproteins oxidizing NADH or NADPH (measured as ferricyanide- or cytochrome *c*-reductase) are very similar in the two varieties. The difference in NADH-cytochrome *c*-reductase activity could be due to a reduced level of cytochrome b_5 in the mutant. It is evident that the electron transport capacity, particularly of the sequence using NADH, is far in excess of that required for 18:1 desaturation. In summary, the enzymatic studies are in agreement with the fatty acid data and support the conclusion drawn above on the possible existence of more than one desaturase activity responsible for 18:1 desaturation in microsomes from sunflower cotyledons.

Acknowledgements

The authors gratefully acknowledge financial support by the BMFT and Fonds der Chemischen Industrie.

Note added in proof: After completion of this manuscript we realized that parallel work on high oleic sunflower (R. Garcés *et al.*, Phytochemistry **28,** 2593 and 2597 [1989]) had come to similar results as described here, so that both investigations complement each other.

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