ORIGINAL ARTICLE

Acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) of *Camelina sativa* seeds: biochemical properties and function

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Abstract

Main conclusion **The transfer of polyunsaturated fatty acids from phosphatidylcholine to other lipids involves several enzymes. In *Camelina sativa* seeds, acyl-CoA:lysophosphatidylcholine acyltransferases could be one of the most important players in this process.**

Abstract The transfer of polyunsaturated fatty acids from the location of their synthesis (phosphatidylcholine) to other lipids, e.g., triacylglycerol, remains insufficiently understood. Several enzymes could be involved in this process. One of these enzymes is acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs). In *Camelina sativa* seeds, LPCATs could be one of the most important players in this process. Our data clearly indicate that the *Cs*LPCATs present in developing seeds have the potential to transfer almost all polyunsaturated fatty acids synthesised on phosphatidylcholine to the acyl-CoA pool. *Cs*LPCAT activity is the highest at 30 °C, and the enzymes operate well at a pH of 7.0–11.0, with the best activity at a pH of 9.0. The activity of *Cs*LPCATs was inhibited by calcium and magnesium ions at a concentration of 0.05–2 mM. In the forward reaction, *Cs*LPCATs preferentially utilise 18:2-CoA; however, other C18 unsaturated fatty acids are also well accepted. In the backward reactions, there is no clear discrimination between the C18 unsaturated fatty acids utilised by the enzymes for phosphatidylcholine remodelling. The activity of *Cs*LPCATs does not differ much between the stages of seed development.

Keywords False flax · Acyl-CoA:lysophosphatidylcholine acyltransferases · Phosphatidylcholine remodelling · Seed lipids · TAG biosynthesis · Triacylglycerol

Abbreviations

DAF	Days after flowering
DTNB	Dithionitrobenzoic acid
FA	Fatty acid
LPC	Lysophosphatidylcholine
LPCAT	Acyl-CoA:lysophosphatidylcholine acyltransferase
LPLAT	Acyl-CoA:lysophospholipid acyltransferase
PC	Phosphatidylcholine
TAG	Triacylglycerol
VLCFA	Very long chain fatty acids
10:0	Capric acid
12:0	Lauric acid
14:0	Myristic acid
16:0	Palmitic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	α-Linolenic acid
20:1	Gondoic acid
22:1	Erucic acid

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Introduction

Camelina sativa L. Crantz (false flax or gold of pleasure), a member of the *Brassicaceae* family, is a relict oil crop that has been cultivated since the Bronze Age. Until World War II, *C. sativa* was one of the most important oilseed crops. However, after the war, false flax was displaced in Europe by other higher-yielding crops such as rapeseed and sunflower

(Zubr 1997). In recent years, interest in *C. sativa* has grown again as its oil represents a potentially new source of essential fatty acids for the human diet, particularly omega-3 fatty acids. Over 50% of the fatty acids in *C. sativa* oil are polyunsaturated fatty acids with a health beneficial ratio between 18:2 and 18:3 (approximately 1:2). This oil also contains a low amount of saturated fatty acids (approximately 10%) and a high content of antioxidant, especially vitamin E (over 70 mg/100 g). All of these factors make *C. sativa* oil an attractive component of the human diet (Zubr 1997; Putnam et al. 1993; Rodriguez-Rodriguez et al. 2013). Additionally, *C. sativa* is a promising oilseed crop for the genetic manipulation of oil content and oil quality, e.g., for industrial use. The low acreage of *C. sativa* cultivation makes it easy to separate eventual transgenic cultivars from non-transformed cultivars.

Acyl-CoA:lysophospholipid acyltransferases (LPLATs) are commonly occurring enzymes in plants, animals and microorganisms. In the forward reaction, LPLATs use lysophospholipids and acyl-CoAs to synthesise the corresponding phospholipids. These enzymes can also transfer fatty acids from phospholipids to the acyl-CoA pool via reverse reactions. Different LPLATs could possess different substrate specificities towards fatty acid acceptors and donors both in their forward and reverse reactions. Depending on the specificity towards the fatty acid acceptor (lysophospholipids), these enzymes take a different names (Shindou et al. 2009; Lager et al. 2013; Jasieniecka-Gazarkiewicz et al. 2016). The enzymes with the highest specificity for lysophosphatidylcholine are called acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs). To date, plant LPCAT activity has been found in microsomal fractions isolated from different plants, e.g., from the developing seeds of safflower (*Carthamus tinctorius* L.) (Ichihara et al. 1995), sunflower (*Helianthus annuus*) (Fraser and Stobart 2000), soybean (*Glycine max* L.) (Tumaney and Rajasekharan 1999) and rapeseeds (*Brasica napus*) (Zheng et al. 2012). The genes encoding LPCAT were first cloned from *Arabidopsis thaliana* in 2008 (Ståhl et al. 2008), and cDNA encoding these enzymes has also been identified in *B. napus* (Zheng et al. 2012), *Nicotiana benthamiana* (Zhang et al. 2015), *Linum usitatissimum* (Pan et al. 2015), *Hiptage benghalensis*, *Lesquerella fendleri*, *Ricinus communis* and *C. tinctorius* (Lager et al. 2013).

In oilseeds, the fatty acids synthesised from acetyl-CoA in the plastids are exported to the cytosol for triacylglycerol (TAG) assembly in the endoplasmic reticulum (ER) (Browse and Somerville 1991). The newly synthesised fatty acids, mainly oleic acid (18:1) and a small amount of palmitic acid (16:0) and stearic acid (18:0), could be either directly used for diacylglycerol (DAG) esterification to produce TAG via the action of diacylglycerol acyltransferase (DGAT) or could be first incorporated into phosphatidylcholine (PC)

for further modifications (Kennedy 1961). The oleic acid in PC is further desaturated to form polyunsaturated linoleic (18:2) and α-linolenic (18:3) acids by the fatty acid desaturases FAD2 and FAD3 associated with the ER. The synthesised polyunsaturated fatty acids in PC could be transferred to the cytosolic pool of acyl-CoA available for TAG synthesis, e.g., via the backward reaction of LPCATs (Lager et al. 2013; Jasieniecka-Gazarkiewicz et al. 2016). The modified fatty acids in PC could also enter the TAG synthesis pathway via the action of CDP-choline:diacylglycerol cholinephosphotransferase (CPT), phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) or phospholipid:diacylglycerol acyltransferase (PDAT). The first two enzymes provide diacylglycerol with modified fatty acids in PC for TAG biosynthesis, and the third enzyme can directly transfer these modified fatty acids from PC to diacylglycerol, producing TAG (Slack et al. 1983; Banaś et al. 2000; Dahlqvist et al. 2000; Bates et al. 2012). The 18:1-CoA exported from plastids could also be elongated to 20:1 and 22:1 by fatty acid elongases before incorporation into TAG (Li-Beisson et al. 2013).

However, thus far, the relative contribution of the enzymes presented above to the transfer of polyunsaturated fatty acids from PC to TAG has not been sufficiently characterised as the activities and substrate specificities of these enzymes have been studied in detail only in a narrow group of selected oilseed crops.

For example, the activity and substrate specificity of the LPCAT type of enzymes have not been studied in *C. sativa*. To bridge this knowledge gap, we characterised the biochemical properties of LPCATs in microsomal fractions from developing *C. sativa* seeds. Moreover, after optimising the assay conditions, we determined the activity and substrate specificity of these enzymes. We have also made predictions regarding the possible contribution of LPCATs to the transfer of polyunsaturated fatty acids from PC to TAG. These predictions were made by comparing the in vivo TAG accumulation intensity and acyl exchange intensity between the PC and acyl-CoA pools obtained from in vitro assays (which allowed us to characterise the backward reaction capacity of LPCATs present in the microsomal fraction from developing *C. sativa* seeds at different stages of development).

Materials and methods

Chemicals

[1-^{14}C]-labelled fatty acids were purchased from Perkin Elmer (Waltham, MA, USA), and non-radioactive fatty acids and *sn*-1-18:1-lysophosphatidylcholine were purchased from Larodan (Malmö, Sweden). Free CoA, bovine

serum albumin (BSA) and heptadecanoic acid methyl ester (17:0-Me) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The standards for thin-layer chromatography were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The acyl-CoAs and [1-^{14}C]-labelled acyl-CoAs were prepared according to the modified method described by Sánchez et al. (1973). The other biochemicals and solvents used for analysis were from Merck (Darmstadt, Germany) or Sigma-Aldrich.

Plant materials

The analyses were performed on *C. sativa* L. Crantz, cv. Suneson. The plants were grown in a growth chamber at a constant temperature of 23 °C with 60% relative humidity and the following long day photoperiod: 16 h of light/8 h of dark at a light intensity of 120 µmol photons $m^{-2} s^{-1}$. Plants with well-developed flower buds were marked, and after 10, 17, 24, 31 and 60 days, parts of their siliques were harvested. The seeds were manually removed from the siliques, and their fresh and dry weights were measured. The freshly harvested seeds were also used to isolate microsomal fractions and for lipid analysis.

Lipid analyses

Lipid extraction from the seeds of *C. sativa* was performed according to the modified methods described by Bligh and Dyer (1959). The seeds were homogenised in Potter–Elvehjem homogeniser with 3.75 ml of chloroform:methanol (1:2; v:v) with subsequent addition of 1.25 ml of 0.15 M acetic acid, 1.25 ml of chloroform and 1.25 ml of water. After vigorous mixing and centrifugation, the bottom chloroform fractions (containing lipids) were collected, dried under a stream of N_2 and dissolved in 1 ml of chloroform.

To analyse the individual lipid classes and determine their fatty acid content and composition, aliquots of the obtained chloroform fractions were separated by thin-layer chromatography on silica gel 60 plates (Merck), using hexane:diethyl ether:acetic acid (70:30:1; v:v:v) or, in the case of PC content analyses, chloroform:methanol:acetic acid:water (90:15:10:2,5; v:v:v:v) as the solvent system. The separated lipid classes were visualised by a brief exposure to I_2 vapours and identified by means of standards. Marked gel fragments containing the appropriate lipid classes were removed and transmethylated in situ on a gel by adding 2 ml of 2% H_2SO_4 in dry methanol (40 min at 90 °C). After incubation, an internal standard (heptadecanoic acid methyl ester) was added together with 3 ml of hexane and 2 ml of water. Following vigorous shaking and centrifugation, the hexane fractions, containing fatty acid methyl esters, were collected and analysed by gas chromatography (Shimadzu; GC-2010) equipped with a flame ionization detector (FID)

and a 60 m × 0.25 mm CP-WAX 58-CB fused-silica column (Agilent Technologies; Santa Clara, CA, USA).

To analyse the fatty acid content and composition of the total acyl lipids present in the chloroform extracts, aliquots of these extracts were dried under a stream of N_2, transmethylated and analysed with GC as described above.

Preparation of microsomal membrane

For the isolation of the microsomal fractions, three stages of seed development were chosen, 17, 24 and 31 DAF, in which it was possible to manually separate the embryo from the seed coat. The collected seed embryos were placed in glass homogeniser and ground after adding 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mg/ml bovine serum albumin, 0.33 M sucrose and catalase (1000 U/ml). The homogenates were filtered through two layers of Miracloth, diluted with fresh extraction buffer to 20 ml and centrifuged at 20,000×*g* for 12 min. The obtained supernatants were collected and centrifuged again at 100,000×*g* for 90 min. The resulting pellets (microsomal fractions) were washed with 0.1 M potassium phosphate buffer (pH 7.2) and homogenised with a small volume of potassium buffer. All stages of microsomal membrane preparation were conducted at 4 °C, and the final isolated fractions were stored at −80 °C until further analysis. To determine the membrane concentrations in the obtained microsomal fractions, aliquots of the suspensions were used for phosphatidylcholine (PC) content analyses.

Enzyme assays

Optimisation tests were carried out to establish the best conditions for the in vitro assays that determined the activity and substrate specificity of the LPCATs from *C. sativa* seeds in forward reactions. Five factors: reaction time, temperature, buffer pH, amount of microsomal fraction and selected ion (K^+, Ca^{2+}, Mg^{2+}) concentration, were analysed. In all of these assays, 5 nmol of exogenous *sn*-1-18:1-lysophosphatidylcholine and 5 nmol of [^{14}C]18:1-CoA were used as exogenous substrates. The results obtained from the preceding tests were included in successive analyses.

After the optimisation step, the activity and substrate specificity of the LPCATs at three different stages of *C. sativa* seed development (17, 24 and 31 DAF) were studied. Ten different [^{14}C]-labelled acyl-CoAs were used: decanoyl-CoA ([^{14}C]10:0-CoA), lauroyl-CoA ([^{14}C]12:0-CoA), myristoyl-CoA ([^{14}C]14:0-CoA), palmitoyl-CoA ([^{14}C]16:0-CoA), stearoyl-CoA ([^{14}C]18:0-CoA), oleoyl-CoA ([^{14}C]18:1-CoA), linoleoyl-CoA ([^{14}C]18:2-CoA), linolenoyl-CoA ([^{14}C]18:3-CoA), eicosenoyl-CoA ([^{14}C]20:1-CoA) and erucoyl-CoA ([^{14}C]22:1-CoA). The reaction mixtures contained 5 nmol of exogenous
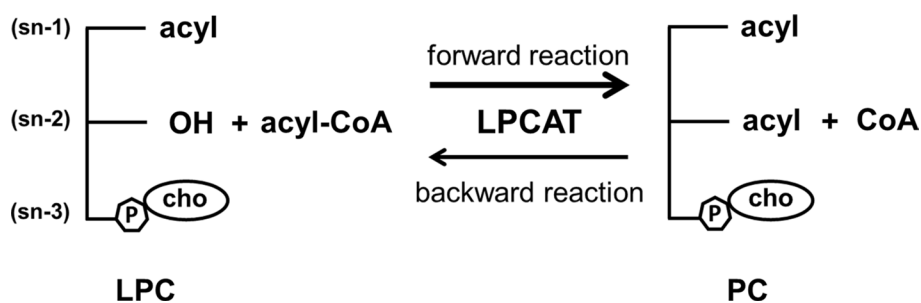
Scheme 1 The reactions catalysed by acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs). In the forward reaction, the synthesis of phosphatidylcholine (PC) is catalysed by the transfer of acyl groups from acyl-CoA to lysophosphatidylcholine (LPC); in the backward reaction, the transfer of acyl groups from PC to coenzyme A (CoA) and synthesis of acyl-CoA takes place

sn-1-18:1-lysophosphatidylcholine, 5 nmol of appropriate [14C]acyl-CoA and aliquots of microsomal fractions equivalent to 0.2 nmol of endogenous PC (approximately 0.88 µg of microsomal proteins) in 100 µl of 40 mM potassium buffer (pH 7.2) or 40 mM HEPES buffer (pH 7.2; assays testing the effects of the selected ion concentrations on LPCAT activity). The reactions were carried out at 30 °C for 30 min with shaking (1250 rpm). The reactions were terminated by the addition of 375 µl of chloroform:methanol (1:2; v:v), 5 µl of glacial acetic acid, 125 µl of chloroform and 125 µl of water. After vigorous shaking, the samples were centrifuged, and chloroform fractions were collected. The extracted lipids present in the chloroform fractions were separated by thin-layer chromatography on silica gel 60 plates (Merck) using chloroform:methanol:acetic acid:water (90:15:10:2,5; v:v:v:v) as the solvent system. The reaction products ([14C]-PC) were visualised and quantified using electronic autoradiography (Instant Imager, Packard Instrument Co.).

In the case of substrate selectivity assays, only the microsomal fractions of *C. sativa* seeds from the "third" stage of development (31 DAF) were used. In these types of assays, five different acyl donors (1 nmol of each) were added to the reaction mixtures (one of the acyl donors was [14C]-labelled). The experiments were performed under two conditions: (i) without the addition of BSA (as in all experiments concerning the forward reaction of *Cs*LPCATs) and (ii) with the addition of BSA, 0.2 mg/assay. All other analysis steps were performed as described above for the assays with only one acyl-CoA in the incubation buffer.

To determine the positional specificity of the LPCATs in the developing seeds of *C. sativa* in forward reactions, the ether analogue of *sn*-1-18:1-LPC, 1-*O*-9-*cis*-octadecenyl-*sn*-glycero-3-phosphocholine (*sn*-1-O-GPC), and the ether analogue of *sn*-2-18:1-LPC, 2-*O*-9-*cis*-octadecenyl-*sn*-glycero-3-phosphocholine (*sn*-2-O-GPC) were used as acyl acceptors together with [14C]18:1-CoA, [14C]18:2-CoA or [14C]16:0-CoA as the acyl donor. The LPC ether analogues in these assays were chosen as the naturally existing

sn-2-LPC (fatty acid is esterified to glycerol backbone) is unstable, and acyl groups rapidly migrate to the *sn*-1 position, contrary to the ether analogue of *sn*-2-LPC (Lager et al. 2013). The microsomal fractions of seeds at 24 DAF were used for the assays. All other analysis steps were performed as described above.

The activity and substrate specificity of LPCATs in the developing seeds of *C. sativa* in backward reactions were examined according to the method described by Jasieniecka-Gazarkiewicz et al. (2016). Incubations were carried out in the presence of aliquots of microsomal fractions corresponding to 5 nmol of endogenous PC (approximately 22 µg of microsomal proteins), 10 nmol of [14C]acyl-CoA, 0.2 µmol of free coenzyme A (CoA) and 1 mg of BSA in a total volume of 100 µl of 40 mM potassium buffer (pH 7.2) with or without 0.5 µmol of dithionitrobenzoic acid (DTNB). Reactions were terminated after 60 min of incubation, and the reaction products were analysed as described above. The activity of the backward reactions catalysed by the LPCATs present in aliquots of the tested microsomal fractions was calculated by subtracting the amount of products synthesised in the assays with DTNB (CoA is bound by DTNB and the backward reaction is stopped) from the amount of products (de novo synthesised [14C]PC) of the assays performed without DTNB (Jasieniecka-Gazarkiewicz et al. 2016; Schemes 1 and 2).

Results

Lipid accumulation in *C. sativa* seeds

Analyses began at 10 DAF and continued at 7-day intervals until 31 DAF. Additional analyses were performed at 60 DAF when the seeds reached complete maturity. The average dry weight of individual seeds at the outset of the analyses was approximately 0.14 mg and increased by approximately 10 times at maturity, achieving 1.4 mg/seed. Between 10 and 31 DAF, the increase in seed dry weight was almost

Scheme 2 Measurement of the backward reaction activity of *Cs*LPCATs in seed microsomal fractions. In each case, two types of assays were performed: one without and one with the addition of DTNB (compound that binds to CoA and makes it unavailable for LPCAT, therefore stopping the backward reaction). Bottom chart presents data from the reactions with microsomal fractions of *C. sativa* seeds at 17 DAF and exogenous [^{14}C]18:1-CoA
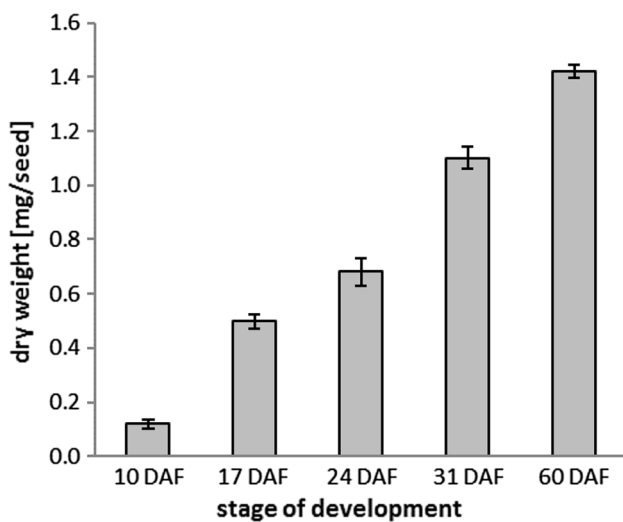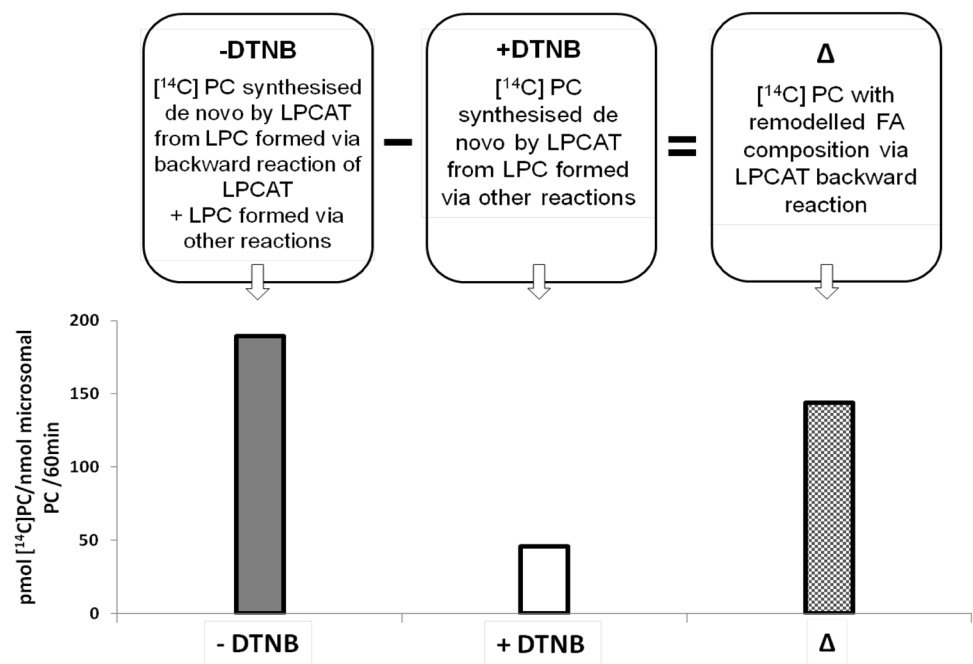

Fig. 1 Development of *C. sativa* seeds during the growing period. The mean values of seed dry weight and SD are presented (data from three biological repetitions with over ten seeds analysed each time)

Fig. 2 Accumulation of total acyl lipids and triacylglycerols (main component of acyl lipids) in developing *C. sativa* seeds. The amount of fatty acids transferred from PC to the acyl-CoA pool via the backward reaction catalysed by *Cs*LPCATs (calculated based on backward reaction activity in vitro) is also presented. Mean values and SD are given ($n \geq 3$)

linear, with the exception of the period between 17 and 24 DAF, when it was slightly slower. Between 31 DAF and maturity, the seed dry weight increased by approximately 0.3 mg——approximately 21% of the final seed dry weight (Fig. 1).

The first samples of developing *C. sativa* seeds were collected for lipid analyses at 10 DAF. At that point in time, the lipid accumulation in seeds was small—below 5% of the final amount of lipids present in mature seeds. Between 10 and 17 DAF, the lipid content in the seeds increased to
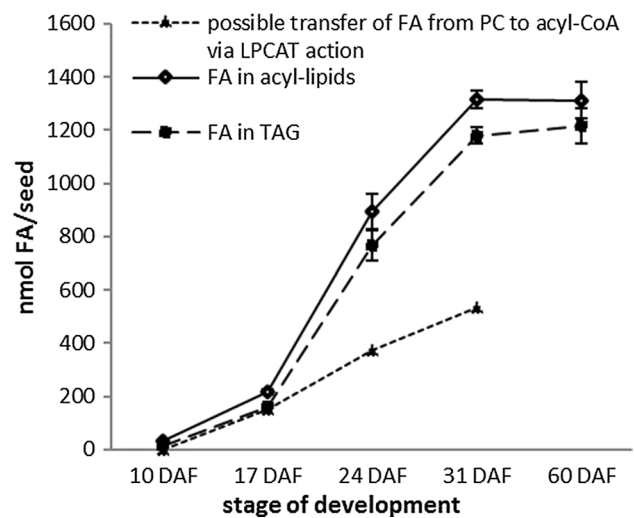
approximately 16% of the final amount. The highest intensity of lipid accumulation occurred between 17 and 24 DAF, when over 50% of lipids were accumulated. Another intensive lipid accumulation process took place between 24 and 31 DAF when over 30% of the total lipids were synthesised. After 31 DAF, there was no net increase in lipid content (Fig. 2).

Table 1 Distribution of lipid classes in developing *C. sativa* seeds

Stage of development	% of total fatty acids					
	Polar lipids	MAG	DAG	FA	TAG	SE
10 DAF	45.5 ± 12.8	1.0 ± 0.1	2.1 ± 0.9	7.5 ± 2.0	42.1 ± 13.2	1.8 ± 0.5
17 DAF	14.3 ± 1.3	0.2 ± 0.01	1.1 ± 0.3	8.0 ± 2.2	75.4 ± 3.6	1.0 ± 0.2
24 DAF	6.4 ± 0.1	0.3 ± 0.01	1.0 ± 0.2	4.5 ± 0.3	86.0 ± 0.1	1.8 ± 0.4
31 DAF	3.6 ± 0.6	0.2 ± 0.1	0.7 ± 0.1	4.2 ± 0.7	89.7 ± 1.8	1.6 ± 0.4
60 DAF	3.7 ± 0.6	0.3 ± 0.05	0.6 ± 0.3	1.4 ± 0.9	92.7 ± 1.9	1.3 ± 0.2

Mean values and SD are presented ($n \geq 3$)

TAG was the dominant lipid class in *C. sativa* seeds, except for the first stage of development. At 10 DAF, the relative amount of TAG was slightly less than the amount of polar lipids and accounted for approximately 42% of the total lipids. At the later stages, the relative amount of TAG gradually increased, reaching approximately 93% of all lipids at maturity. The amount of polar lipids was relatively high at the early stages of seed development (approximately 45% of total lipids) but accounted for only 3.7% of the total lipids in the mature seeds. The relative amount of DAG ranged from 2.1% (10 DAF) to 0.6% (mature seeds). The relative amount of free fatty acids decreased from approximately 7.5% (10 and 17 DAF) to 1.4% (mature seeds) of the total lipids. The amount of sterol esters fluctuated between 1 and 1.8%, with no clear trend among the analysed stages of seed development. MAG contents were the lowest among all lipid classes and ranged between 0.2 and 0.3% of the total lipids (except for stage 1–10 DAF, where MAG accounted for approximately 1% of the total lipids) (Table 1).

Despite the decrease in the relative amount of polar lipids in *C. sativa* developing seeds over time, their absolute amount increased until 24 DAF. Afterwards, the absolute amount decreased, especially between 24 and 31 DAF. Phosphatidylcholine—the major phospholipid—accounted for approximately 53% of the total polar lipids. The changes in the absolute amount of PC in the developing seeds reflect the changes in total polar lipids (Fig. 3).

The analysis of the fatty acid composition of the total acyl lipids present in chloroform extracts of *C. sativa* seeds revealed thirteen different types of them. Only five of these fatty acids (16:0; 18:0; 18:1; 18:2 and 18:3) were observed in detectable amounts at 10 DAF, and all the remaining fatty acids began to appear in the later stages of seed development. Six of them (20:0, 20:2, 20:3, 22:0, 24:0, 24:1) were present only in small amounts, and they were analysed together as "other" fatty acids (Table 2). Linoleic (18:2) and linolenic (18:3) acids were the dominant fatty acids throughout the entire seed development period. However, the relative amount of 18:2 decreased from approximately 44% at 10 DAF to approximately 18% at seed maturity, while conversely, 18:3 levels increased from approximately 12%
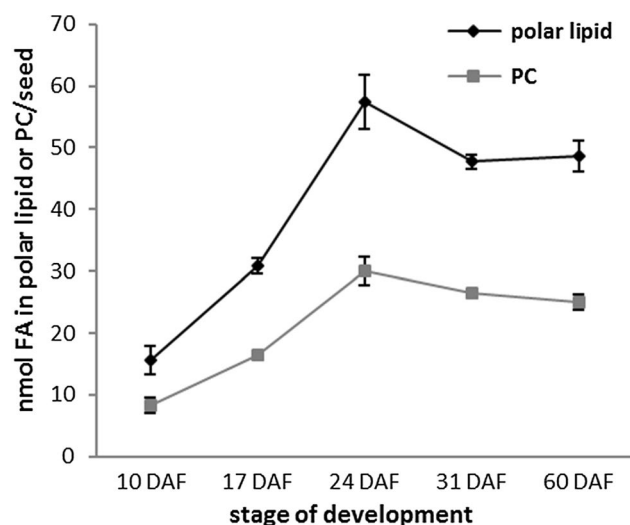

Fig. 3 The content of polar lipids and phosphatidylcholine (PC) in developing *C. sativa* seeds. Mean values and SD are presented ($n \geq 3$)

to approximately 37%. Monounsaturated oleic acid ($18:1^{\Delta 9}$) and eicosenoic acid ($20:1^{\Delta 11}$) were the second most prevalent group of fatty acids in terms of the amount found in *C. sativa* seeds. The highest amount of 18:1 was at 17 DAF (23%), and 18:1 accounted for approximately 12% of all the fatty acids in mature seeds. The relative amount of 20:1 accounted for approximately 5% at 17 DAF and 13–15% in other stages of seed development. The relative amount of saturated fatty acids decreased throughout seed development: 16:0 decreased from approximately 23% (10 DAF) to 7.5% at maturity and 18:0 decreased from approximately 6% to approximately 3%. Erucic acid ($22:1^{\Delta 13}$) was detected at 17 DAF (0.5%), and its level increased during seed development to approximately 3% in mature seeds. The amount of "other" fatty acids also increased during seed development from 2.4% (17 DAF) to 6.6% in mature seeds (Table 2). The fatty acid composition of the analysed lipid classes reflected, to a certain degree, the composition of fatty acids described above (data not presented). Nevertheless, in the "polar lipid" class, erucic acid and "other" fatty acids were not present, and the relative amount of 20:1 ranged only between 2 and 4%. The relative amount of 16:0 was high (ranging from 17

Table 2 Fatty acid composition of the acyl lipids in developing *C. sativa* seeds

Stage of development	FA (mol%)							
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	Other
10 DAF	23.1 ± 3.3	6.4 ± 1.3	14.8 ± 1.5	43.8 ± 2.7	11.9 ± 1.0			
17 DAF	11.5 ± 0.7	7.0 ± 0.2	23.3 ± 0.2	35.7 ± 0.1	14.4 ± 0.1	5.2 ± 0.2	0.5 ± 0.02	2.4 ± 0.1
24 DAF	8.6 ± 0.2	4.3 ± 0.2	14.1 ± 1.2	25.4 ± 0.6	26.8 ± 0.8	13.3 ± 0.3	2.3 ± 0.3	5.2 ± 0.5
31 DAF	8.7 ± 0.2	3.4 ± 0.2	10.2 ± 0.4	19.6 ± 1.2	33.5 ± 1.5	14.9 ± 0.2	3.1 ± 0.1	6.6 ± 0.2
60 DAF	7.5 ± 0.1	2.7 ± 0.2	12.0 ± 0.2	18.4 ± 0.5	37.2 ± 0.8	12.7 ± 0.3	2.9 ± 0.1	6.6 ± 0.1

Other: 20:0, 20:2, 20:3, 22:0, 24:0, 24:1

Mean values and SD are presented ($n \geq 3$)

Table 3 Fatty acid composition of the polar lipids in developing *C. sativa* seeds

Stage of development	FA (mol%)					
	16:0	18:0	18:1	18:2	18:3	20:1
10 DAF	22.9 ± 4.6	3.8 ± 0.1	11.5 ± 0.2	46.8 ± 2.3	15.0 ± 2.2	
17 DAF	19.4 ± 3.5	3.9 ± 1.1	12.3 ± 1.2	35.5 ± 2.1	26.6 ± 1.9	2.3 ± 0.5
24 DAF	24.3 ± 2.4	4.3 ± 0.2	8.2 ± 1.5	25.7 ± 0.7	34.4 ± 0.4	3.1 ± 0.03
31 DAF	17.5 ± 0.3	2.3 ± 0.04	7.4 ± 0.1	25.6 ± 0.4	45.1 ± 0.7	2.1 ± 0.1
60 DAF	17.3 ± 0.8	5.5 ± 0.1	22.7 ± 1.4	24.6 ± 1.1	25.7 ± 3.7	4.2 ± 0.2

Mean values and SD are presented ($n \geq 3$)

to 24%), and the level of 18:3 had substantially decreased between 31 DAF (45%) and maturity (26%) (Table 3).

In vitro activity of *C. sativa* LPCATs

In the experiments, [^{14}C]18:1-CoA and *sn*-1-18:1-LPC were used as exogenous substrates for LPCATs and the microsomal fractions of *C. sativa* seeds at 24 DAF were used as a source of enzymes. In the preliminary assays under conditions adapted from earlier studies (Lager et al. 2013; Jasieniecka-Gazarkiewicz et al. 2016), we showed that the reaction rate catalysed by the LPCATs in the analysed microsomal fractions was almost linear up to 30 min of incubation. These preliminary results were confirmed in further assays under optimised assays conditions (Fig. 4a).

Aliquots of microsomal fractions containing 0.2 nmol of microsomal PC (approximately 0.88 µg of microsomal proteins) were optimal for the assays. Either higher or lower amounts of these fractions resulted in lower enzyme activity (Fig. 4d). The optimal temperature for the LPCATs of *C. sativa* seeds was shown to be 30 °C. Nevertheless, enzyme activity was rather high at temperatures ranging between 20 and 40 °C. Decreasing the temperature to 10 °C reduced the enzyme activity to approximately 1/3 of the maximum level. Increasing the temperature to 50 °C almost inactivated the enzymes; their activity plunged to approximately 1/7 of the

maximal level. At 60 °C, the enzyme activity was negligible (Fig. 4b).

Four buffers were used to determine the effect of pH: 0.1 M phosphate buffer (pH 5.5–8.0); 0.1 M Tris–HCl buffer (pH 8.0–10.0); 0.1 M NaHCO$_3$–NaOH buffer (pH 10.0 and 11.0); and 0.1 M NaHPO$_4$–NaOH buffer (pH 11.0 and 12.0). *C. sativa* LPCATs were inactive at a pH of 5.5, and up to a pH of 6.0, their activity increased only marginally (1/10 of the maximum activity). The enzymes operated fairly efficiently at a pH of 7.0–11.0. The maximum activity of LPCATs was observed at a pH of 9.0 (Tris–HCl buffer). Above that level, LPCAT activity decreased to approximately 64% at a pH of 11.0 (NaHCO$_3$–NaOH buffer) and to approximately 34% of its maximal value at a pH of 12.0 (Na$_2$HPO$_4$–NaOH buffer) (Fig. 4c).

The LPCAT activity of *C. sativa* seeds was also affected by the concentration of Mg^{+2}, Ca^{+2} and K$^+$ ions. In these assays, HEPES buffer was used instead of phosphate buffer (in which Mg^{+2} and Ca^{+2} ions form insoluble salts). The addition of magnesium and calcium ions to the incubation buffer at concentrations as low as 0.05 mM inhibited LPCAT activity by 27–34%. An increase in the tested ion concentrations further strengthened the LPCAT inhibition. However, the inhibition rate was not linearly correlated with increase in ion concentrations, at least in the case of Mg^{+2} and Ca^{+2}. At a concentration of 2 mM of the tested ions in the assay buffer, the inhibition rate ranged from approximately 28%
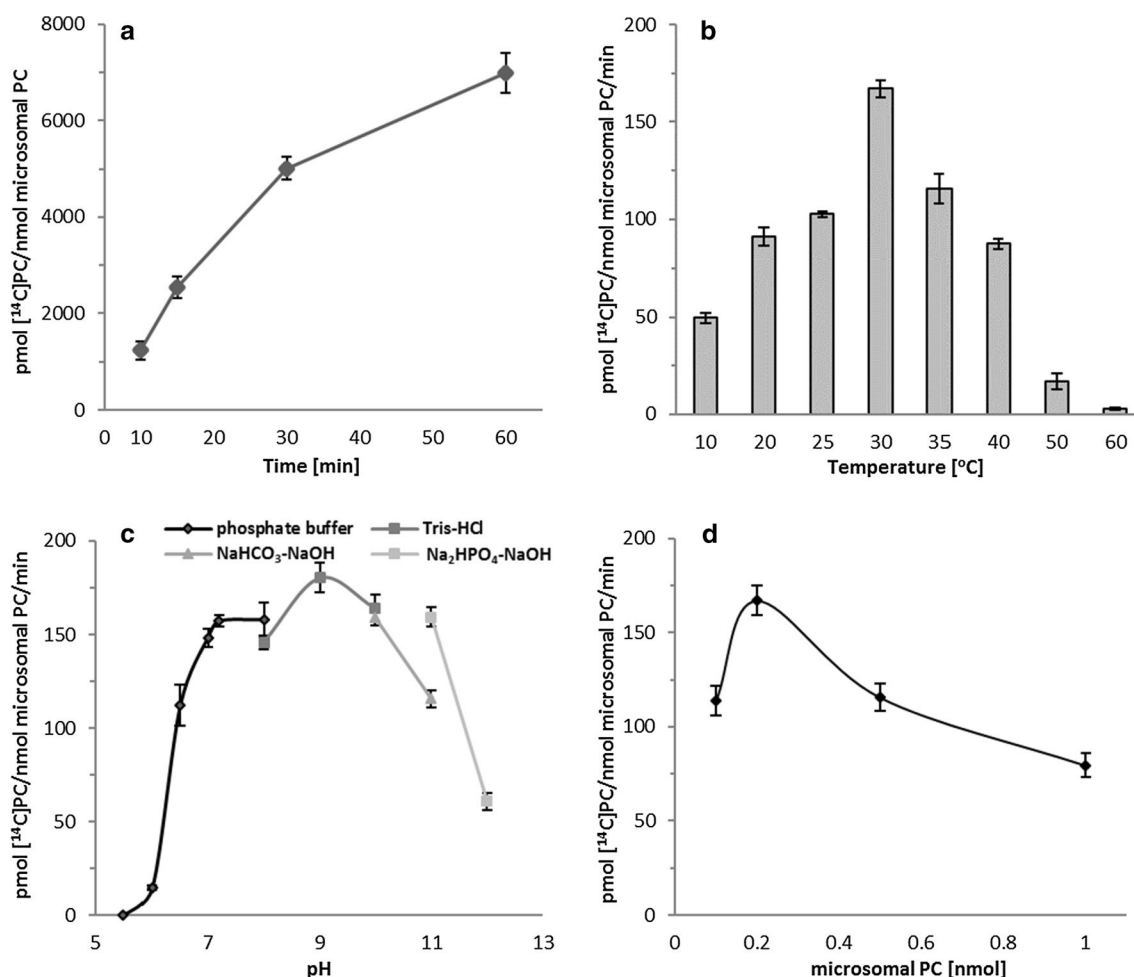
Fig. 4 The effect of various factors on the activity of acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) of *C. sativa* seeds. **a** Time dependency. **b** Temperature dependency. **c** pH depend-

ency. **d** Microsomal content dependency. Mean values and SD are presented (data from at least three independent assays)

in the case of K$^+$ to 53% (Mg^{+2}) and approximately 62% (Ca^{+2}) (Fig. 5).

The LPCATs in *C. sativa* seed microsomal fractions were rather stable enzymes. Preincubation of microsomal fractions at 30 °C for 60 min reduced LPCAT activity by approximately 38% and preincubation for approximately 15 min reduced the activity by only approximately 16% (Fig. 6).

Heating the tested microsomal fractions for 10 min at 100 °C completely destroyed all LPCAT activity (data not presented).

Activity and substrate specificity of the LPCATs in developing *C. sativa* seeds in forward reactions

LPCAT activity was measured in microsomal fractions prepared from *C. sativa* seeds at 17 DAF, 24 DAF and 31 DAF, i.e., at the time points of seed development when lipids were the most intensively accumulated. In the assays, ten

different [^{14}C]acyl-CoAs in combination with *sn*-1-18:1-LPC were used as exogenous substrates. For the majority of the tested substrate combinations, the highest LPCAT activity was observed in the microsomal fractions prepared from the seeds at 24 DAF (except for 10:0-CoA and 18:0-CoA, which showed the lowest activity at this time point, and 20:1-CoA and 22:1-CoA, which were not accepted by *Cs*LPCATs). Nonetheless, the LPCAT activity in the two other stages of seed development was also high and usually amounted to over 50% of the levels observed at 24 DAF (Fig. 7). The most preferred acyl donor in the assays with the microsomal fraction of the 24 and 31 DAF seeds was 18:2-CoA (at 17 DAF 18:3-CoA was preferred). The maximum LPCAT activity with this acyl-CoA reached approximately 210 pmol [^{14}C]PC/nmol of microsomal PC/min (equal to 52 nmol [^{14}C]PC/mg microsomal protein/min). The two other unsaturated 18C-acyl-CoAs (18:1 and 18:3) were also well accepted by the enzymes. The efficiency of *Cs*LPCATs
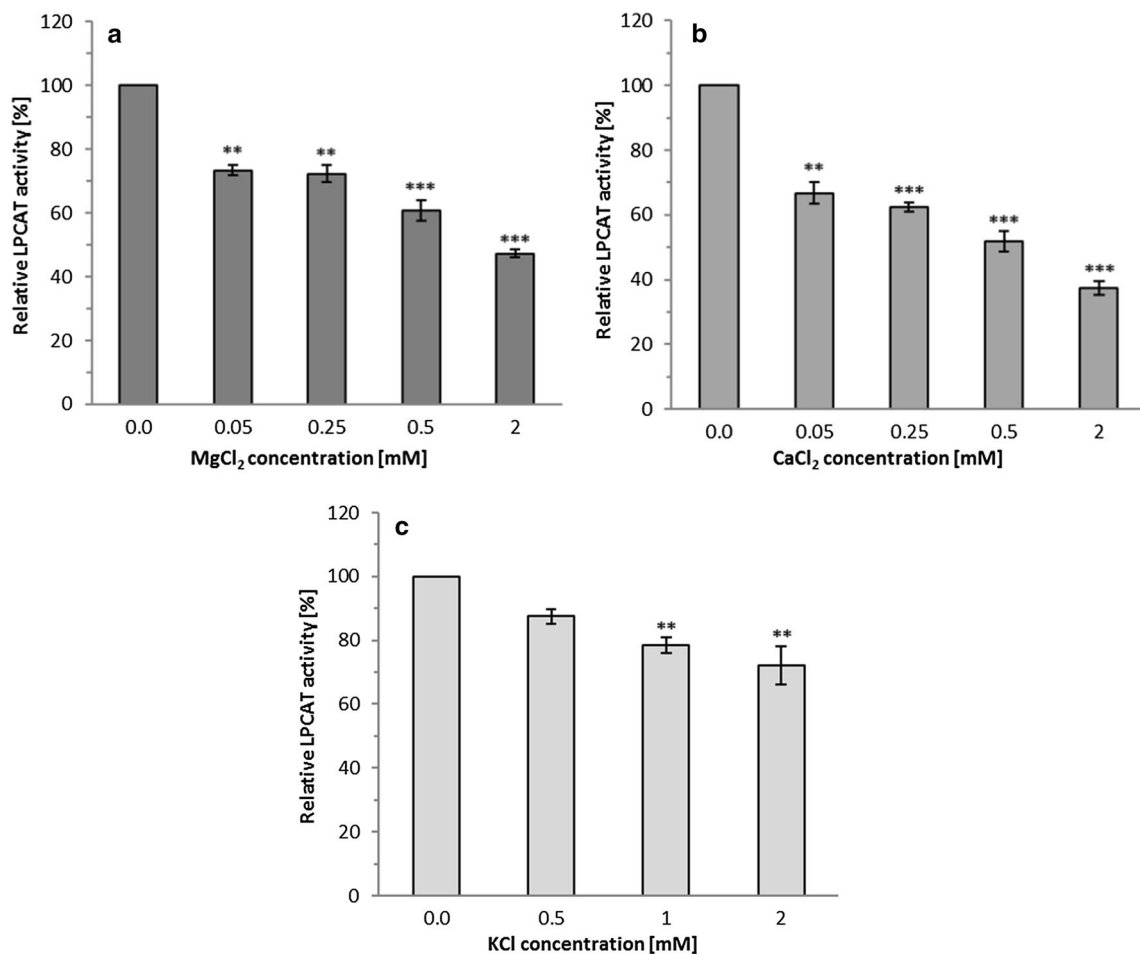
Fig. 5 The effect of various ions on the activity of acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) of *C. sativa* seeds. **a** Effect of magnesium ions. **b** Effect of calcium ions. **c** Effect of potassium ions. Mean values and SD are presented (data from at least three independent assays). Asterisks denote significant differences between the control (ions not added) and the tested ion concentrations in a mean difference two-sided Student's t test: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$

towards 18:1-CoA and 18:3-CoA was between 60 and 130% of its activity towards 18:2-CoA depending on the developmental stage of the seed. 16:0-CoA and 18:0-CoA were utilised with efficiencies ranging from approximately 12–28% and 4–12% of activity towards 18:2-CoA, respectively. The enzyme also utilised 10:0-CoA, 12:0-CoA and 14:0-CoA, however, with a very low activity (below 6% of its activity towards 18:2-CoA) (Fig. 7).

The substrate selectivity assays were performed to verify whether the preferences of *Cs*LPCATs towards different acyl-CoAs observed in the assays described above were similar when different acyl-CoAs were in the reaction mixture. In these assays, five different acyl-CoAs (1 nmol of each) were added to the reaction mixtures (one acyl-CoA was [^{14}C]-labelled), and all other steps of the analysis were carried out in the same way as in the case of the assays with only one acyl-CoA. The results showed that the preferences of *Cs*LPCATs towards different acyl-CoAs added as

an equimolar mixture were very similar to those obtained in the assays with only one acyl-CoA. 18:2-CoA was the most favoured substrate, whereas 18:3-CoA and 18:1-CoA were utilised with approximately 30% lower efficiency. The incorporation of acyl groups from 16:0-CoA and 18:0-CoA was relatively weak (approximately 6 and 1% of activity towards 18:2-CoA, respectively), similar to the single acyl-CoA assay (Fig. 8a). The addition of BSA to the incubation buffer at a concentration of 0.2 mg/assay slightly increased the utilisation of 16:0-CoA, 18:0-CoA and 18:1-CoA compared to 18:2-CoA (to 16%, 6% and 80%, respectively) and decreased the utilisation of 18:3-CoA to approximately 45% of the utilisation of 18:2-CoA (Fig. 8b).

The assays with the ether analogue of sn-1-18:1-LPC (*sn-1-O-GPC*) and the ether analogue of *sn-2-18:1-LPC* (*sn-2-O-GPC*) showed that the *Cs*LPCATs in seed microsomal fractions can acylate both LPC positions. However, the efficiency of *Cs*LPCATs towards the *sn-2* position was approximately 8
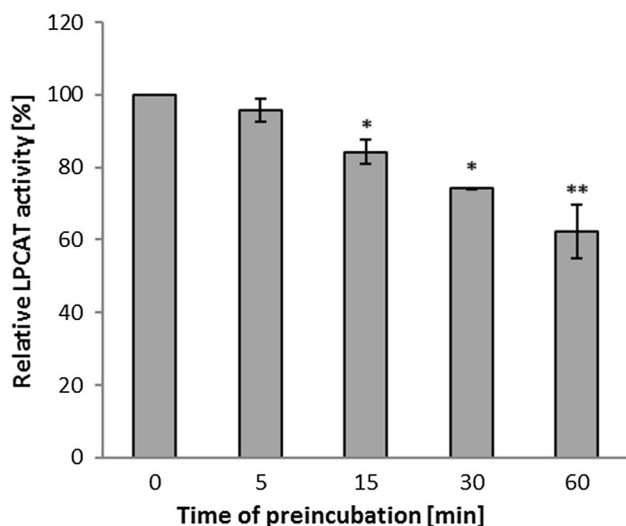
Fig. 6 The effect of preincubation time on the activity of the acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) in *C. sativa* seeds. Mean values and SD are presented (data from at least three independent assays). Asterisks denote significant differences between the activity of the *Cs*LPCATs in the non-preincubated microsomal fraction (control) and those preincubated for different times at 30 °C in a mean difference two-sided Student's *t* test: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

times higher than that towards the *sn*-1 position when 18:1-CoA and 18:2-CoA were used as fatty acid donors and approximately 1.6 times higher when 16:0-CoA was used (Fig. 9).

Activity of the LPCATs in developing *C. sativa* seeds in backward reactions

Similar to the measurement of *Cs*LPCAT activity in the forward reactions, we also measured the activity of these

enzymes in the backward reactions using microsomal fractions prepared from *C. sativa* seeds at 17 DAF, 24 DAF and 31 DAF. In these assays, three [14C]acyl-CoAs (18:1-CoA, 18:2-CoA and 18:3-CoA) were used as fatty acid donors for remodelling endogenous/microsomal PC. Reactions were carried out with and without the addition of DTNB. We assumed that in both types of reactions, the incorporation of [14C]acyl groups into PC would occur via the acylation of endogenous LPC. However, the endogenous LPC in assays with DTNB was created only via reactions other than the LPCAT backward reactions (DTNB binds CoA and the backward reaction was inhibited), e.g., PDAT or lipase activity, while in assays without DTNB, LPC was synthesised in the mentioned above "other reactions" and additionally via the backward reaction of LPCATs. Thus, by subtracting the amount of de novo synthesised [14C]PC in assays with DTNB from the amount of [14C]PC synthesised in the assays without DTNB, we obtained the activity of the backward reaction of LPCATs (Jasieniecka-Gazarkiewicz et al. 2016, Scheme 2).

In our assays, the activity of the backward reactions of the *Cs*LPCATs in seed microsomal fractions was generally the highest at 17 DAF. There was a clear difference in the reaction intensities among the analysed stages of seed development when 18:1-CoA was used as a fatty acid donor for PC remodelling. At 24 and 31 DAF, the activity was 60 and 35% of the activity at 17 DAF, respectively. At 17 DAF, 18:1-CoA was also the best acyl donor. At 24 and 31 DAF, the best acyl donor for PC remodelling was 18:2-CoA. In all analysed stages of seed development, 18:3-CoA was the worst acyl donor. The backward activity with 18:3-CoA as a fatty acid donor declined slightly with
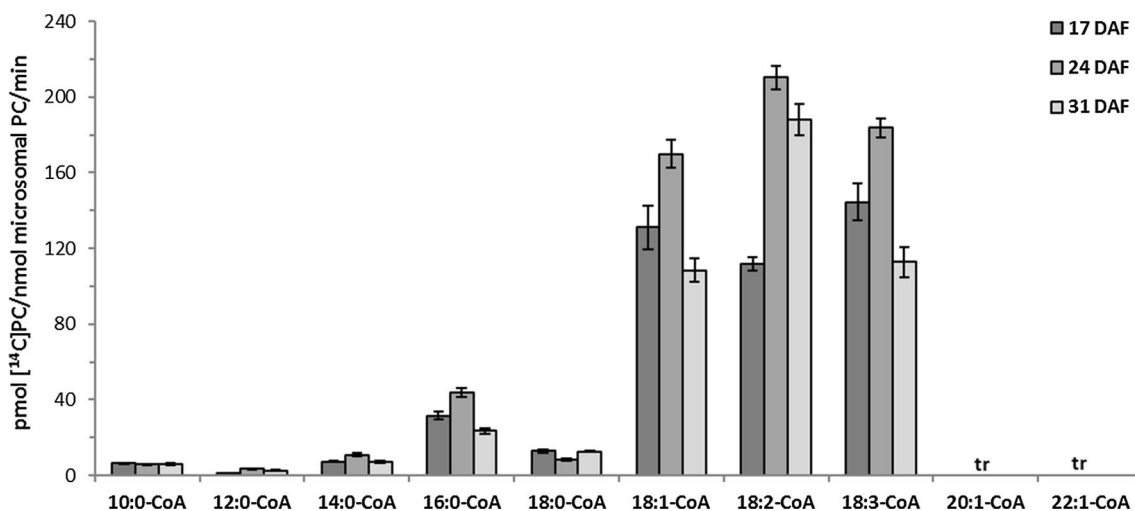

Fig. 7 Activity of the acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) in *C. sativa* seeds towards ten different acyl-CoAs (forward reaction). Mean values and SD are presented (data from at least three independent assays); *tr* trace level
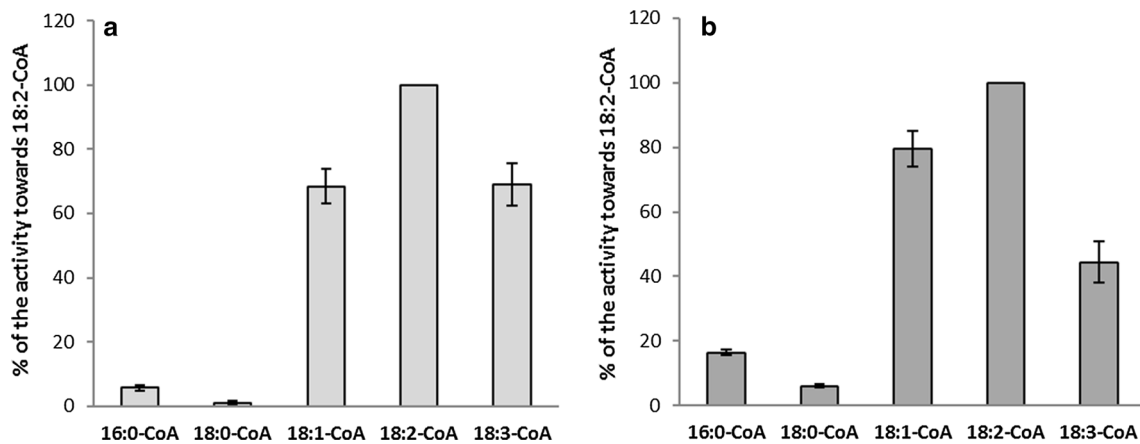
Fig. 8 Activity of the acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) in *C. sativa* seeds towards five different acyl-CoAs added to the reaction mixture together in equimolar concentrations without (**a**) or with (**b**) the addition of BSA (forward reaction; substrate selectivity assay). For the assays, the microsomal fractions of 31 DAF seeds were used. Mean values and SD are presented (data from at least three independent assays)
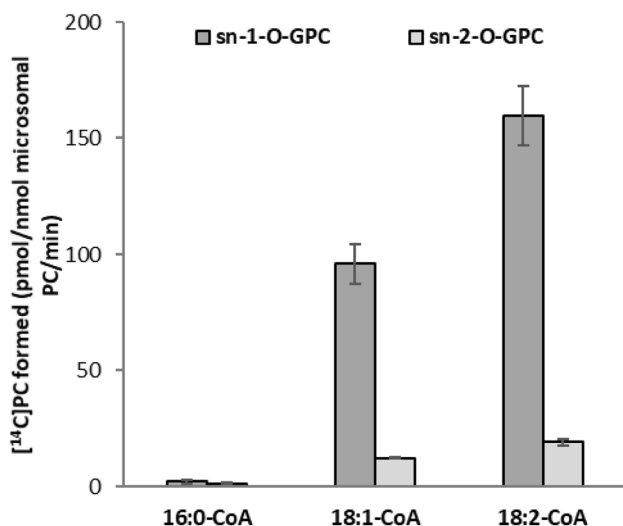

Fig. 9 Activity of *C. sativa* LPCATs towards the ether analogue of *sn*-1-18:1-LPC (*sn*-1-18:1-O-GPC) and the ether analogue of *sn*-2-18:1-LPC (*sn*-2-18:1-O-GPC) with $[^{14}C]16:0$-CoA, $[^{14}C]18:1$-CoA and $[^{14}C]18:2$-CoA as acyl donors. For the assays, the microsomal fractions of the seeds from the stage with the highest activity of LPCATs (24 DAF) was used. Mean values and SD are presented (data from at least three independent assays)

Table 4 Incorporation of $[^{14}C]$acyl groups from $[^{14}C]$acyl-CoA into PC in the microsomal fractions of *C. sativa* seeds in the presence and absence of DTNB

Stage of development	Acyl-CoA used in the assay	pmol $[^{14}C]$PC/nmol microsomal PC/min		Δ
		− DTNB	+ DTNB	
17 DAF	$[^{14}C]18:1$-CoA	3.16 ± 0.4	0.76 ± 0.1	2.4
	$[^{14}C]18:2$-CoA	2.65 ± 0.5	0.91 ± 0.05	1.74
	$[^{14}C]18:3$-CoA	1.78 ± 0.05	0.78 ± 0.1	1.0
24 DAF	$[^{14}C]18:1$-CoA	2.04 ± 0.2	0.64 ± 0.01	1.4
	$[^{14}C]18:2$-CoA	2.16 ± 0.1	0.72 ± 0.06	1.44
	$[^{14}C]18:3$-CoA	1.39 ± 0.05	0.46 ± 0.04	0.93
31 DAF	$[^{14}C]18:1$-CoA	1.8 ± 0.1	0.95 ± 0.05	0.85
	$[^{14}C]18:2$-CoA	2.56 ± 0.06	0.94 ± 0.06	1.62
	$[^{14}C]18:3$-CoA	1.51 ± 0.1	0.65 ± 0.06	0.86

Delta represents the incorporation with DTNB subtracted from the incorporation without DTNB and represents the incorporation via acyl exchange (LPCAT backward reaction)

Mean values and SD are presented (data from at least three independent assays)

seed development time, while there was no clear tendency in the case of 18:2-CoA (Table 4).

In the backward reactions catalysed by LPCATs, apart from LPC, an equimolar amount of acyl-CoA is created (the acyl moiety comes predominantly from the *sn*-2 position of PC). Thus, the capacity of this reaction (measured as described above) also indicates the capacity of the remodelling of the acyl-CoA pool available for different acyltransferases (Lager et al. 2013; Jasieniecka-Gazarkiewicz et al. 2016). Considering this relationship, we calculated the

potential amount of polyunsaturated fatty acids that could be transferred from PC to the acyl-CoA pool (via the backward reactions catalysed by *Cs*LPCATs) to make them available for other processes, e.g., TAG synthesis. In this calculation, we used the capacity of the backward reaction obtained in the assays with $[^{14}C]18:1$-CoA as fatty acid donors for PC remodelling (pmol $[^{14}C]$PC/nmol microsomal PC/min; Table 4), the amount of nmol PC/seed (Fig. 3) and the time between the analysed stages of seed development. For the period between 10 and 17 DAF, we took the backward reaction activity of LPCATs at 17 DAF and the average value of PC content/seed at 10 and 17 DAF (as seeds at 10 DAF were

not used for microsomal preparation). For the calculations between 17 and 24 DAF and between 24 and 31 DAF, we used the average values of the LPCAT backward reaction activity and the average amount of PC/seed between these stages. We obtained the potential amount of fatty acids that could be transferred from PC to the acyl-CoA pool between the analysed stages of seed development by multiplying the average value of the backward reaction activity by the average amount of PC/seed (which indicated the amount of enzymes in the whole seed) and by the time between the analysed stages. The calculated potential transfer of fatty acids from PC to the acyl-CoA pool via the backward reactions of *Cs*LPCATs was high and ranged from approximately 100% (early stages of development) to approximately a half (later stages of development) of the total amount of fatty acids accumulated in triacylglycerols in *C. sativa* developing seeds (Fig. 2). The relative amount of polyunsaturated fatty acids in the TAG of mature *C. sativa* seeds is approximately 55%. Thus, the capacity of the backward reaction of *Cs*LPCATs present in developing seeds should be sufficient to transfer these fatty acids from the place of synthesis (PC) to the acyl-CoA pool available for TAG biosynthesis. It should be noted, however, that these estimations represent only a theoretical possibility of fatty acid transfer from PC to the acyl-CoA pool and are based on the backward reaction capacity of *Cs*LPCATs determined with in vitro assays.

Discussion

It has been postulated since the 1980s that LPCATs play a role in acyl exchange between PC and the acyl-CoA pool via the backward reaction (Stymne and Glad 1981; Stymne and Stobart 1984). However, the fact that LPCATs can operate in both the forward and backward directions has been definitively proven only recently (Lager et al. 2013; Pan et al. 2015; Jasieniecka-Gazarkiewicz et al. 2016). To date, the capacity of the backward reactions of LPCATs in the developing seeds of oilseed plants has not been specified; therefore, it was not possible to establish to what extent they could be responsible for the transfer of polyunsaturated fatty acids from PC to the acyl-CoA pool. In this study, we successfully determined the backward reaction capacity of LPCATs in microsomal fractions from *C. sativa* seeds at different developmental stages. By evaluating enzyme activity in aliquots of microsomal fractions containing 1 nmol of PC and by estimating the PC content in individual seeds at each analysed stage of development, we were able to calculate the entire enzyme activity in the seeds and consequently the amount of fatty acids that could be transferred from PC to the acyl-CoA pool throughout seed development. The obtained data clearly indicate that the *Cs*LPCATs present in developing seeds have the potential to transfer almost all

the polyunsaturated fatty acids (half of total fatty acids) synthesised in PC to the acyl-CoA pool. This statement does not imply that LPCATs outcompete other enzymes in vivo (see Introduction) that are involved in the transfer of the modified fatty acids in PC to other lipids, e.g., TAG. This finding means only that in *C. sativa* seeds, LPCATs could be important or major players in this process. *Cs*LPCATs do not discriminate between polyunsaturated fatty acids and oleic acid for remodelling PC. Thus, under in vivo conditions, the relative availability of 18:1-CoA and other acyl-CoAs for *Cs*LPCATs will determine what kind of PC is synthesised. However, as over 55% of all TAG fatty acids are polyunsaturated, 18:1 has to be efficiently incorporated into PC and undergo the desaturation process. This study did not evaluate the acyl-CoA profile of developing *C. sativa* seeds. However, previous studies (Ruiz-Lopez et al. 2016) have shown that the acyl-CoA pool of *C. sativa* seeds at 28 DAF (which roughly corresponds to a period between the second—24 DAF—and the third—31 DAF—stage of seed development in our study) contains approximately 2.6 times more 18:1-CoA than 18:2-CoA and approximately 1.5 times more 18:1-CoA than 18:3-CoA. The good utilisation of 18:2-CoA and 18:3-CoA for PC remodelling was a surprising result. At the outset, we assumed that the physiological function of this process is a transfer of polyunsaturated fatty acids from the place of biosynthesis (PC) to the acyl-CoA pool available for different lipid biosynthesis/remodelling (Scheme 3). One of the possible explanations of this phenomenon could be that the 18:1-CoA available for elongation by the cytosolic elongation system comes not only directly from plastids (the place of synthesis) but possibly from the PC pool (Bao et al. 1998). The improved utilisation of 18:2-CoA compared with 18:1-CoA for PC remodelling at 24 DAF and 31 DAF of seed development than at 17 DAF correlates well with the higher rate of biosynthesis of very long chain fatty acids observed at later stages of seed development. At 17 DAF, the total amount of VLCFA accounted for approximately 8% of the total fatty acids of seed acyl lipids, whereas at 31 DAF, it accounted for more than 25%. The capacity of the backward reaction catalysed by *Cs*LPCATs does not correlate well with the capacity of the forward reaction. The backward reaction activity of *Cs*LPCATs was the highest at 17 DAF, and the forward reaction activity was the highest at 24 DAF. Previously, it has also been shown that LPCAT activity in the forward reaction is not correlated with its activity in the backward reactions (Jasieniecka-Gazarkiewicz et al.2016).

Storage lipids accumulated in *C. sativa* seeds mainly between 17 and 31 DAF. This finding was consistent with the suggestion that up to 14–17 days from flowering, seeds mainly increase the number of their cells ("growth phase"), and only after this period does the "accumulation phase" occur (Rodriguez-Rodriguez et al. 2013). The mature seeds of *C. sativa* contained approximately 80% unsaturated fatty

Scheme 3 Biosynthetic pathway of 18C unsaturated fatty acids and the role of acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs) in the transfer of these fatty acids to the acyl-CoA pool available for lipid biosynthesis (e.g., TAG) or for remodelling and elongation by the cytosolic elongation system. Desaturation and elongation processes as well as lipid biosynthesis and remodelling take place in the endoplasmic reticulum. *FAD2* fatty acid desaturase 2, *FAD3* fatty acid desaturase 3, *TAG* triacylglycerol
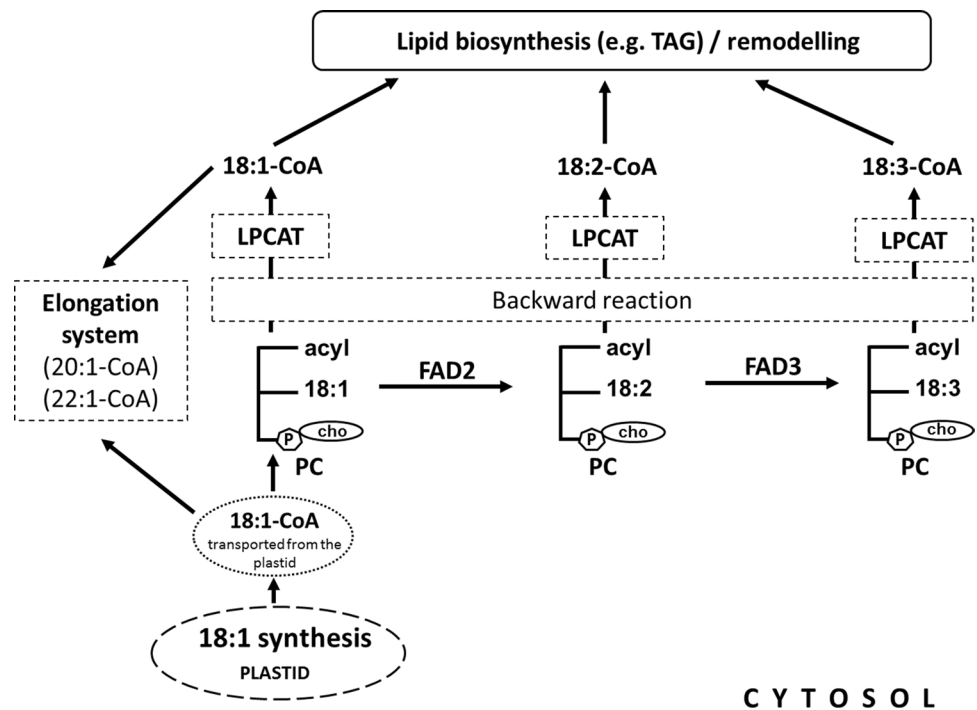
acids (18:3, 37%: 18:2, 18%; 20:1, 13%; and 18:1, 12%, approximately). Similar fatty acid compositions of acyl lipids of *C. sativa* seeds have also been reported by others (Zubr 1997; Gugel and Falk 2006; Rodriguez-Rodriguez et al. 2013; Marmon et al. 2017). After 31 DAF, the net increase in the acyl lipids in *C. sativa* seeds was not recorded. However, at that time, the relative amount of TAG increased by approximately 3%. Additionally, at that time period, the relative amount of 18:3 in the acyl lipids of *C. sativa* seeds (of which approximately 93% were TAG) increased by approximately 3%. At the same time, the relative amount of 18:3 in the "polar lipid" class decreased by approximately 21%. Thus, one can propose that at this stage of seed development, the synthesis of TAG occurs via the remodelling of the acyl lipids present in the seeds, and some kind of exchange of fatty acids between polar and storage lipids occurs and the desaturation process can still occur.

The LPCATs of *C. sativa* seeds operate with the highest activity at a pH of 9.0–10.0, i.e., under conditions that do not exist in the natural environment of these enzymes. However, this could be a common feature for the LPCATs in different organisms. For instance, the LPCATs of *B. napus* have the highest activity at a pH of 10.0 (Oo and Huang 1989; Furukawa-Stoffer et al. 2003), and the same is true in the case of LPCAT1 present in the lungs of mice (Nakanishi et al. 2006). *Cs*LPCATs were inhibited by Mg^{2+} and Ca^{2+} ions and, to a certain extent, by K^+ ions. In the case of magnesium and calcium, the inhibition was not linearly correlated with the tested ion concentrations. This finding could suggest that different mechanisms of

inhibition occurred at low and high concentrations of these ions. At concentrations of both Mg^{2+} and Ca^{2+} as low as 0.05 mM, it could be speculated that the inhibition was directly connected to the modification of the enzyme structure caused by these ions. Such a concentration could be the saturation point for the enzyme, as even a fivefold increase in ion concentration did not further change the enzyme activity. Any further decrease in LPCAT activity could be explained by the influence of other types of tested ions. For instance, it was shown that Mg^{2+} ions at concentrations above 5 mM could affect acyl-CoA solubility (Constantinides and Steim 1986). The total Mg^{2+} concentration in cells ranges from 15 to 25 mM (Moomaw and Maguire 2008). However, most Mg^{2+} ions are bound or incorporated into cellular components, which leaves only approximately 0.4–0.5 mM as free cytosolic Mg^{2+} (Karley and White 2009; Maathuis 2009). Even that concentration could already affect the acyl-CoA solubility, nevertheless, it must be noted that in cells, acyl-CoAs are bound by acyl-CoA-binding protein, which also influences solubility (Yurchenko et al. 2009). Thus, the results from in vitro studies cannot be directly implemented under in vivo conditions. The inhibition of the LPCATs in microsomal fractions of rat submandibular glands and rat heart activity by 10 mM Ca^{2+} and Mg^{2+} has previously been reported (Soupene et al. 2008). The inhibition of *Cs*LPCATs by K^+ was not as spectacular as the inhibition caused by magnesium and calcium (10 and 30% inhibition at 0.5 mM and 2 mM, respectively); however, plant cells maintain a relatively high K^+ concentration (100–200 mM) in the

cytosol (Blumwald et al. 2000). Thus, in nature, potassium ions could have some effects on LPCAT activity.

The positional specificity of the *Cs*LPCATs of seed microsomal fractions does not differ much from that of Arabidopsis LPCATs. Lager et al. (2013), in experiments similar to those performed in this study, proved that *At*LPCAT1 and *At*LPCAT2 show approximately 7–8 times higher activity towards the *sn*-2 position than towards the *sn*-1 position of LPC when 18:1-CoA is the fatty acid donor and approximately 3–5 times higher in cases when 16:0-CoA is the fatty acid donor. Thus, both studies indicate that LPCATs preferentially acylate the *sn*-2 position of LPC and can acylate the *sn*-1 position, with much lower efficiency. The LPCATs in the microsomal fractions of *C. sativa* seeds showed a clear preference for 18C unsaturated acyl-CoA in the forward reaction. Similar substrate specificity of the LPCATs in the microsomal fractions of yeast with mutated main endogenous acyl-CoA:lysophospholipid acyltransferase (ALE1) overexpressed with genes encoding the LPCATs of *A. thaliana, L. fendleri, C. tinctorius, R. communis, H. benghalensis* and *L. usitatissimum* were previously shown (Lager et al. 2013; Pan et al. 2015). Thus, it seems that the LPCATs in oilseed plants do not discriminate between 18:2-CoA and 18:3-CoA in favour of 18:1-CoA. This should be expected, as 18:1 undergoes a desaturation process after incorporation into PC. Thus, similar to our conclusions on the utilisation of acyl-CoA for PC remodelling connected with the backward reaction of LPCATs, the availability of 18:1-CoA (which is, according to Ruiz-Lopez et al. 2016, higher than that of polyunsaturated acyl-CoA), 18:2-CoA and 18:3-CoA most likely decides what kind of PC is synthesised via LPCAT action. The preferences of *Cs*LPCAT for different acyl-CoAs obtained in assays with single acyl-CoA were confirmed in substrate selectivity assays where five different acyl-CoAs were added to the reaction mixtures in equimolar concentrations. However, under in vivo conditions, the presence of acyl-CoA binding proteins (ACBPs) could affect the preferences of LPCATs for different acyl-CoAs. Thus, in one variation of the substrate selectivity assays, we added BSA to the reaction buffer (in in vitro assays, the ACBPs are often replaced by BSA) at a concentration of 2 mg/ml. The concentration of BSA used was probably in excess of the concentration of ACBPs present in the cells. Engeseth et al. (1996) showed, for example, that in the tissues of *A. thaliana,* the concentrations of ACBPs ranged between 3 and 143 μg/g FW. The obtained results were quite similar to those obtained in the experiments without the addition of BSA (only a slight modification of the preferences of *Cs*LPCATs towards different acyl-CoA were noted). This finding indicates that the preferences of *Cs*LPCATs towards different acyl-CoA measured in our in vitro assays could also be similar to those under in vivo conditions. According to

Ruiz-Lopez et al. (2016), a substantial amount of the cytosolic acyl-CoA pool consists of very long chain fatty acid-CoAs. For instance, 20:1-CoA constitutes approximately 19.5% and 22:1-CoA constitutes approximately 10% of this pool. However, these fatty acids are observable only in trace amounts in PC (data not presented). This result correlates well with the very low affinity of *Cs*LPCATs for these acyl-CoAs. Thus, contrary to 18C-acyl-CoA, the substrate specificity of *Cs*LPCATs determines the presence of these fatty acids in PC only in trace amounts.

The activity of *Cs*LPCAT was the highest at 24 DAF; however, it was also quite high at 17 DAF and 31 DAF. The observed LPCAT activity was probably generated by different isoforms of *Cs*LPCATs. The data in the genome database of *C. sativa* indicate the presence of six *Cs*LPCAT isoforms. Three of these isoforms probably encode LPCAT1, and the other three probably encode LPCAT2. However, this assumption was made only based on the similarity of the genes encoding these isoforms to the genes encoding the LPCATs of *A. thaliana*. To date, no experimental data have confirmed that these genes truly encode *Cs*LPCATs. Preliminary data on the expression levels of these candidate genes of *Cs*LPCATs were presented by Abdullah et al. (2016). This data showed that between the first stage of seed development (10–15 DAF) and the second stage (16–21 DAF), the expression levels of all candidate genes encoding *Cs*LPCAT isoforms increased. This finding is in agreement with our results showing an increase in *Cs*LPCAT activity between these stages of seed development. The acylation rate of LPC to form PC in our assays using the crude microsomal fraction could be a result of not only *Cs*LPCAT activity but also of *Cs*LPEAT (acyl-CoA:lysophosphatidylethanolamine acyltransferases) activity, as it was shown earlier that LPEAT enzymes can also acylate LPC to some degree (Jasieniecka-Gazarkiewicz et al. 2016). In our preliminary assays, we have shown, however, that the activity of *Cs*LPEAT in seed microsomal fractions accounted for only approximately 10% of the activity of *Cs*LPCAT. Thus, the effects of endogenous *Cs*LPEATs on the obtained results concerning the activity and substrate specificity of *Cs*LPCATs seem to be negligible.

Author contribution statement SK, KJ-G and AB conceived and designed the research. SK and KJ-G conducted the experiments. SK, KJ-G and AB analysed the data and wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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