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Lipid biosynthesis in developing perilla seeds

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Abstract

In developing seeds of *Perilla frutescens* var. *crispa*, the triacylglycerol fraction was found to accumulate between 15 and 19 days after flowering. Of this, 65% of the total fatty acids was α-linolenic acid in the mature seeds, with the latter being esterified in comparable amounts at all positions (*sn*-1, 2 and 3) of the glycerol residue. It was also demonstrated that, 1-acylglycerol-3-phosphate acyltransferase, which catalyzes esterification at the *sn*-2 position of the glycerol backbone, showed low activities for α-linolenoyl-CoA as substrate. These findings suggest that the diacylglycerol precursor for triacylglycerol synthesis is not directly derived from phosphatidic acid through the glycerol phosphate pathway.

Keywords: *Perilla frutescens*; Lamiaceae; α-Linolenic acid; Triacylglycerol; 1-Acylglycerol-3-phosphate acyltransferase

1. Introduction

The ratio of *n*-6 to *n*-3 polyunsaturated fatty acids in the human diet substantially effects the *n*-6/*n*-3 ratios in various tissues, and hence an imbalance in this ratio deleteriously affects various diseases and brain functions (Lands, 1993; Leonard, 1999; Connor, 2000). α-Linolenic acid, an *n*-3 trienoic acid, is an essential fatty acid for humans, and only the developing seeds of limited plant species produce this fatty acid in large quantities. Perilla oil is not a major vegetable oil. However, it is one of important oil resources, because more than 60% of the total fatty acids in triacylglycerol (TG) is α-linolenic acid (Hilditch, 1956). In the plant cell, α-linolenate is synthesized by desaturation of linoleate esterified in glycerolipid molecules of membranes (Browse and Somerville, 1991). The site of the ω3 desaturase reaction is the endoplasmic reticulum or chloroplasts (Heinz, 1993), and a seed-specific ω3 desaturase cDNA of perilla has recently been cloned and characterized (Chung et al., 1999). Additionally, mutants deficient in α-linolenate synthesis were isolated in linseed and *Arabidopsis* (Stymne et al., 1992; Browse et al., 1993).

In general, TG is synthesized de novo from glycerol 3-phosphate (GP) and acyl-CoAs by the combination of transacylation and dephosphorylation.

$$\mathrm{GP} \xrightarrow{\text{acyl-CoA}} \text{1-acyl-GP} \xrightarrow{\text{acyl-CoA}} \mathrm{PA} \xrightarrow{-\mathrm{Pi}} \mathrm{DG} \xrightarrow{\text{acyl-CoA}} \mathrm{TG}$$

However, the mechanism by which TG rich in α-linolenate is synthesized in seed cells is not clear. 1-Acyl-GP acyltransferase (EC 2.3.1.51) catalyzes the second acylation in the GP pathway. This acyltransferase enzyme is responsible for the fatty acid composition at the *sn*-2 position of the phosphatidic acid (PA) product and in turn for the composition at the *sn*-2 position of *sn*-1,2-diacylglycerol (DG) which is the direct precursor of TG (Frentzen, 1993). DG is also formed from phosphatidylcholine (PC) via the reverse reaction of DG cholinephosphotransferase (EC 2.7.8.2) [DG + CDP-choline ← → PC + CMP] (Slack et al., 1983, 1985). PC is formed either by the forward reaction of cholinephosphotransferase or by the action of 1-acylglycerophosphocholine (1-acyl-GPC) *O*-acyltransferase or lysolecithin acyltransferase (EC 2.3.1.23) [1-acyl-GPC + acyl-CoA ⟷ PC + CoA] (Stymne and Stobart, 1984). TG is also synthesized by transacylation between two molecules of DG or between DG and phospholipids (Stobart et al., 1997; Dahlqvist et al., 2000). The reaction of monoacylglycerol acyltransferase (Tumaney

Abbreviations: DG, *sn*-1, 2-diacylglycerol; GP, glycerol 3-phosphate; GPC, glycerophosphocholine; PA, phosphatidic acid; PC, phosphatidylcholine; TG, triacylglycerol.

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et al., 2001) or phospholipase C results in the formation of DG, but their participation in TG synthesis is questionable.

Here, we report on the profiles of lipid accumulation and changes in the fatty acid composition in developing perilla seeds. The origin of DG as the precursor of TG synthesis is also discussed on the basis of the positional distribution of α-linolenic acid in TG molecules and the substrate specificity of 1-acyl-GP acyltransferase.

2. Results and discussion

2.1. Accumulation of TG rich in α-linolenate during seed development

In developing perilla seeds, lipids were rapidly accumulated between 15 and 19 days after flowering (Fig. 1). The accumulated lipids were predominantly composed of TG. Fig. 1 shows that TG synthesis of perilla seeds occurs in a very limited, short period of development. During this period, the dry weight per seed increased from 0.44 to 0.94 mg, which was close to that of a matured seed, 1.06 mg. The lipid content of mature seeds 31 days after flowering was 27% of the dry weight. After the rapid synthesis of lipids, the α-linolenic acid content reached 64–65% of the total fatty acids (Fig. 2). In TG molecules of both developing and mature seeds, palmitic and stearic acids were exclusively localized at the *sn*-1 and 3 positions, whilst α-linolenic acid was present in comparable amounts at all positions, through somewhat higher at the *sn*-2 position (Table 1). According to the current concept of TG synthesis in plants, the fatty acid composition of each position in TG reflects the acyl-CoA composition in the cell and the acyl-CoA specificity or selectivity of the corresponding acyltransferase responsible for the acylation of each position in the glycerol moiety (Harwood, 1997). 1-Acyl-GP acyltransferase involved in the GP pathway catalyzes the esterification of the *sn*-2 position of the glycerol backbone, and the microsomal acyltransferases from some plant species are specific to unsaturated acyl-CoAs including α-linolenoyl-CoA (Bafor et al., 1990). Because saturated acyl-CoAs are, in general, poor substrates for the reaction, only unsaturated fatty acids are found at the *sn*-2 position of TG. However, considerable amounts of saturated fatty acids are present as constituents of glycerolipids in the cell. The distribution profile of saturated and unsaturated acyl moieties at the *sn*-2 position does not therefore appear to represent the probable fatty acid composition of the acyl-CoA pool in the cell, because saturated fatty acids are excluded from this position. This means that, in TG molecules that are synthesized exclusively via the GP pathway, the fatty acid composition of the *sn*-2 position depends both on the acyl-CoA specificity of the acyltransferase enzyme and on the composition of unsaturated fatty acyl-CoAs. In developing perilla seeds, however, the activity of 1-acyl-GP

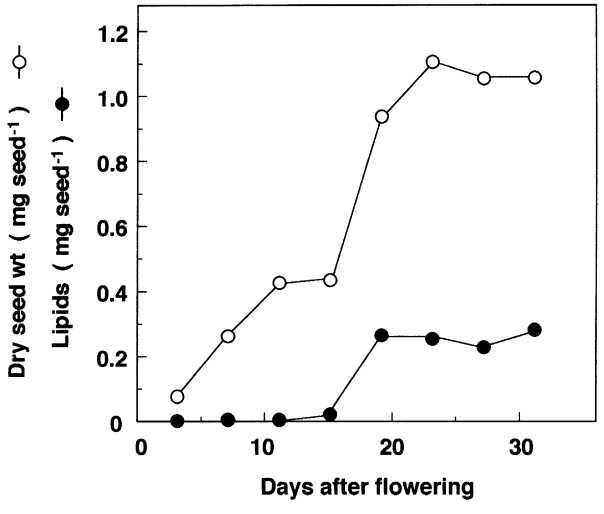

Fig. 1. Changes in seed wt and lipid content during seed development.

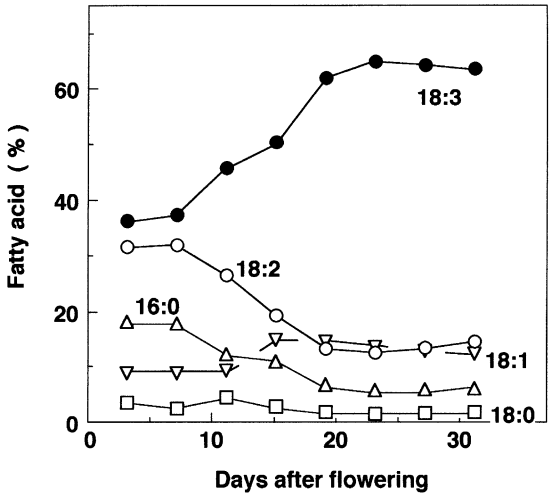

Fig. 2. Changes in the fatty acid composition of glycerolipids during seed development. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid.

Table 1
Positional distribution of fatty acids in perilla TG

Developing stage (DAF)	Position	Fatty acid (%)				
		16:0	18:0	18:1	18:2	18:3
19	*sn*-1,3	10.3	3.0	13.7	10.8	62.2
	sn-2	0.0	0.0	16.9	16.2	67.0
	Total	6.9	2.0	14.8	12.6	63.8
31	*sn*-1,3	8.2	2.8	13.7	12.5	62.8
	sn-2	0.0	0.0	12.9	17.8	69.3
	Total	5.5	1.9	13.4	14.3	64.9

DAF, days after flowering. For abbreviations of fatty acids, see the legend of Fig. 2.

acyltransferase was relatively low for α-linolenoyl-CoA (Fig. 3). In the range of lower concentrations, α-linolenoyl-CoA was the most inert acyl donor among the acyl-CoAs tested. It is noteworthy that the acyltransferase activity was lower for α-linolenoyl-CoA than for palmitoyl-CoA, which is a typical saturated acyl-CoA. Palmitic acid was absolutely localized at the *sn*-1 and *sn*-3 positions, while no palmitic acid was distributed at the *sn*-2 position of TG (Table 1). At a high concentration of 50 μM of acyl-CoA, the reaction rate for α-linolenoyl-CoA remained low. Although substrate specificity for acyl-CoA molecular species is not necessary to reflect substrate selectivity exactly, the PA product of the 1-acyl-GP acyltransferase reaction in vivo is expected to have a relatively low content of α-linolenate at the *sn*-2 position. These data suggest that perilla TG rich in α-linolenate is, at least, not directly synthesized through sequential reactions of the GP pathway, GP→1-Acyl-GP→PA→DG→TG. A possibility can not be excluded that an unknown factor regulates the acyltransferase activity for a specific acyl-CoA, α-linolenoyl-CoA, in vivo, but no such factor has been found so far.

Another pathway that supplies the DG precursor of TG is the reverse reaction of cholinephosphotransferase, PC + CMP→DG + CDP-choline (Slack et al., 1983; Stobart and Stymne, 1985). PC is the site of desaturation of oleate and also of linoleate (Stymne and Appelqvist, 1978). The acyl moiety of oleoyl-CoA is incorporated into the *sn*-2 position of lysoPC by the action of 1-acyl-GPC acyltransferase, which is specific to unsaturated acyl-CoAs (Fig. 4). The oleoyl residue of PC is desaturated to linoleate by microsomal Δ12 desaturase and then further to α-linolenate by microsomal ω3 desaturase (Cherif et al., 1975; Heinz, 1993). While these polyunsaturated fatty acids that have been formed by desaturases are released from the membrane PC to the cytosol in the form of CoA derivatives, PC can be also split into DG and CDP-choline in the presence of CMP by cholinephosphotransferase. In perilla seeds, α-linolenate-containing DGs thus synthesized are likely to serve as the precursor of TG. It was reported that PC was not a major phospholipid class in mature seeds of *P. frutescens* var. *japonica* but a minor membrane constituent, and that major phospholipids were phosphatidylethanolamine and phosphatidylinositol (Noda and Obata, 1975). The unusual phospholipid composition of perilla seeds may be associated with the high α-linolenate content in TG and with the very low activity for α-linolenoyl-CoA in the acyltransferase reaction of PA synthesis. The low PC content of perilla seeds may be a consequence of the accelerated conversion of membrane PC into TG.

The data presented is here consistent with the current concept of TG synthesis in developing oilseeds that accumulate polyunsaturated fatty acids (Frentzen, 1983). 1-Acyl-GPC acyltransferase, oleate desaturase and linoleate desaturase are involved in a metabolic cycle to incorporate oleate into the cycle, to desaturate it to α-linolenate and to release the trienoic fatty acid as the CoA derivative. In developing perilla seeds, the PC desaturation/acyl-exchange cycle is presumably set in the classical GP pathway for the production of polyunsaturated fatty acids. α-Linolenoyl-CoA that has been released from the desaturation/acyl-exchange cycle is a major component of the acyl-CoA pool in the cell, and it is utilized for the first and third acylations of the glycerol backbone by GP and DG acyltransferases,

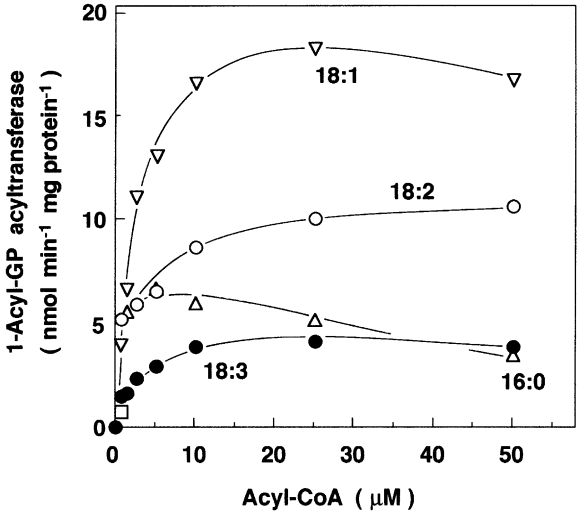

Fig. 3. Dependence of the 1-acyl-GP acyltransferase reaction on the acyl-CoA concentration. For abbreviations of fatty acyl residues, see the legend of Fig. 2.

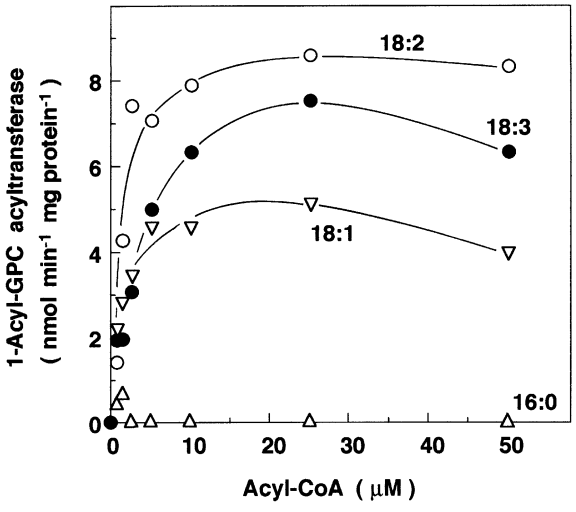

Fig. 4. Dependence of the 1-acyl-GPC acyltransferase reaction on the acyl-CoA concentration. For abbreviations of fatty acyl residues, see the legend of Fig. 2.

respectively. Part of polyunsaturated PC molecules that have been formed by the desaturation system in the cycle supply the DG precursor for the synthesis of TG containing α-linolenate at the *sn*-2 position without undergoing the acyl-exchange reaction (Slack et al., 1978). This modified GP pathway probably plays a major role in the overall process of TG synthesis in developing perilla seeds.

When most of the storage TG is synthesized via PC and not through the classical GP pathway (Fig. 5), acyl-exchange between oleoyl-CoA and the α-linolenoyl moiety at *sn*-2 of PC by 1-acyl-GPC acyltransferase in the cycle can be omitted once (corresponding to acylation at *sn*-2) in three times per molecule of tri-α-linolenoylglycerol. Because linoleoyl-CoA that has been released from PC (2-linoleoyl) can be incorporated into the desaturation/acyl-exchange cycle again, the cycle must operate faster than the typical rate of TG synthesis to form three molecules of α-linolenoyl-CoA per TG molecule. No acyl exchange is necessary for the synthesis of 1-palmitoyl-2-α-linolenoyl-3-oleoyl glycerol through the modified GP pathway in which the glycerol backbone of TG is derived from PC (Fig. 5B). Perilla seeds may save the acyl-exchange reaction to increase the α-linolenate content of TG to the observed value of 65% of the total fatty acids.

3. Experimental

3.1. Reagents

Oleic and linoleic acids, 99% pure, were kindly provided by Nippon Oil & Fats Co. (Amagasaki, Japan). Other fatty acids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Nu-Chek Prep (Elysian, MN, USA). Acyl-CoAs were prepared by a mixed anhydride method and their concentrations were determined spectrophotometrically (Ichihara and Noda, 1982). 1-Oleoyl-GP and 1-oleoyl-GPC were enzymatically prepared from PC (1,2-dioleoyl) that had been chemically synthesized from GPC and oleic anhydride (Ichihara et al., 1987).

3.2. Plant material and lipid analysis

Perilla frutescens var. *crispa*, which is called "shiso" in Japan, was used in this study, although commercial perilla oil is extracted from *P. frutescens* var. *japonica*, "egoma". Perilla plants were grown in the University field and maturing seeds were harvested at desired stages after flowering. Lipids were extracted with $CHCl_3$/MeOH (1:1) and determined gravimetrically. Fatty acid components of the total glycerolipids were converted into methyl

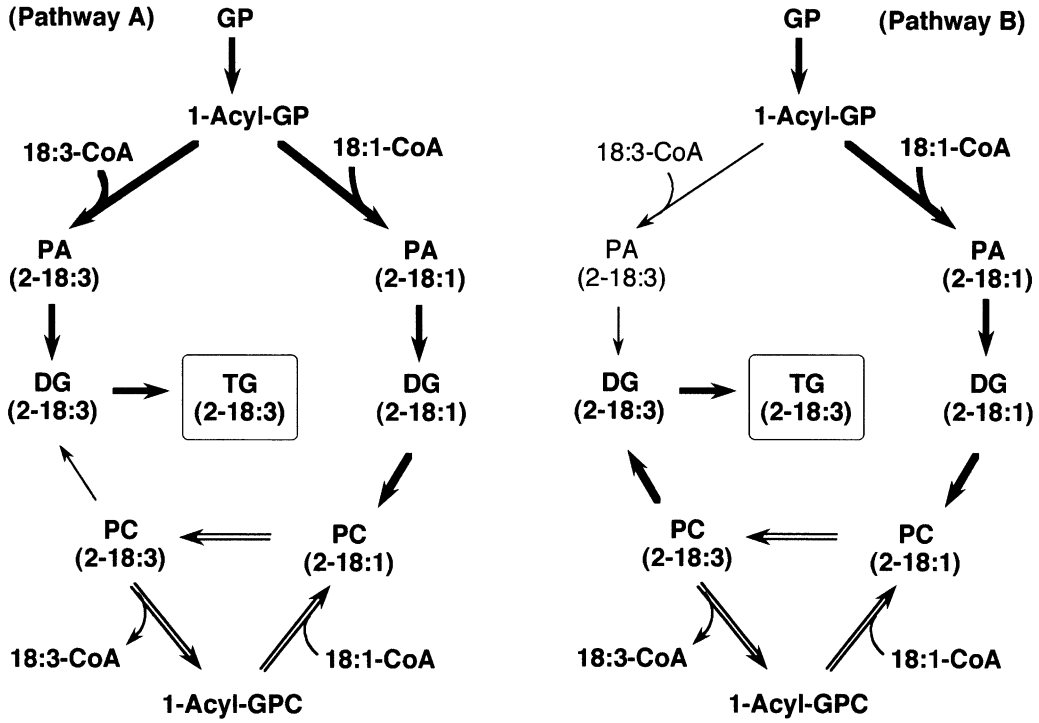

Fig. 5. Two possible pathways for TG synthesis in perilla seeds. Pathway A: TG (2-α-linolenoyl) is mainly synthesized from PA (2-α-linolenoyl) via the GP pathway, which is independent of the desaturation cycle (⇒). The α-linolenoyl-CoA substrate for PA synthesis is supplied from the desaturation cycle. Little PC (2-α-linolenoyl) is converted into DG (2-α-linolenoyl). Pathway B: TG (2-α-linolenoyl) is mainly synthesized from PC (2-α-linolenoyl) through the desaturation system. The desaturation/acyl-exchange cycle supplies α-linolenoyl-CoA for acylation of the *sn*-1 position of GP and the *sn*-3 position of DG. 1-Acyl-GP is mainly esterified with oleoyl-CoA or linoleoyl-CoA to give PA. The abbreviations, 2-18:1 and 2-18:3 mean oleoyl and α-linolenoyl at *sn*-2, respectively.

esters by transesterification with KOH/MeOH (Ichihara et al., 1996) and analyzed by GC (4 mm×2 m glass columns packed with 15% DEGS, isothermal 190 °C, N_2 30 ml min^{-1}). The positional distribution of fatty acids in TG was determined by the method of digestion with pancreatic lipase (Mattson and Volpenhein, 1961).

3.3. Preparation of microsomes and enzyme assay

Microsomes were prepared from developing seeds 19 days after flowering. The seeds were homogenized in 10 mM Mops–NaOH (pH 7.2) containing 0.4 M sorbitol, 0.5 mM EDTA and 2.5% insoluble polyvinylpolypyrrolidone. The homogenate was centrifuged at 3000 g for 10 min, and then the supernatant was centrifuged at 20,000 *g* for 1 h. The precipitate was homogenized in 10 mM Mops–NaOH (pH 7.2) containing 0.4 M sorbitol and 0.5 mM EDTA. The membrane suspension thus prepared was stored at −80 °C until use. Protein was determined by the method of Lowry et al. (1951).

The 1-acyl-GP acyltransferase activity was assayed in 1 ml 150 mM Tris–HCl buffer (pH 8.0) containing 0.4 M sorbitol, 0.25 mM EDTA, 2 mM spermidine, 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid), 10 mM oleoyl-CoA, 50 mM 1-oleoyl-GP, and 50 μg microsomal protein (Ichihara et al., 1987). The assay medium for 1-acyl-GPC acyltransferase activity was the same as described above, except that spermidine was not contained and that 1-oleoyl-GP was replaced by 1-oleoyl-GPC. Each assay is based on the reduction of the disulfide reagent by the sulfhydryl group of CoA liberated, and increase in absorbance at 412 nm was continuously followed at 30 °C for 1 min.

References

Bafor, M., Stobart, A.K., Stymne, S., 1990. Properties of the glycerol acylating enzymes in microsomal preparations from developing seeds of safflower (*Carthamus tinctorius*) and turnip rape (*Brassica campestris*) and their ability to assemble cocoa-butter type fats. J. Am. Oil Chem. Soc. 67, 217–225.

Browse, J., McConn, M., James Jr., D., Miquel, M., 1993. Mutants of Arabidopsis deficient in the synthesis of α-linolenate. J. Biol. Chem. 268, 16345–16351.

Browse, J., Somerville, C., 1991. Glycerolipid synthesis: biochemistry and regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 467–506.

Cherif, A., Dubacq, J.P., Mache, R., Oursel, A., Tremolieres, A., 1975. Biosynthesis of α-linolenic acid by desaturation of oleic and linoleic acids in several organs of higher and lower plants and algae. Phytochemistry 14, 703–706.

Chung, C.-H., Kim, J.-L., Lee, Y.-C., Choi, Y.-L., 1999. Cloning and characterization of a seed-specific ω-3 fatty acid desaturase cDNA from *Perilla frutescens*. Plant Cell Physiol. 40, 114–118.

Connor, W.E., 2000. Importance of n-3 fatty acids in health and disease. Am. J. Clin. Nutr. 71, 171S–175S.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., 2000. Phospholipid:DG acyltransferase: an enzyme that catalyzes the acyl-CoA indendent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. USA 97, 6487–6492.

Frentzen, M., 1993. Acyltransferases and triacylglycerols. In: Moore Jr., T.S. (Ed.), Lipid Metabolism in Plants. CRC Press, Boca Raton, pp. 195–230.

Harwood, J.L., 1997. Plant Lipid Metabolism. In: Dey, P.M., Harborne, J.B. (Eds.), Plant Biochemistry. Academic Press, San Diego, pp. 237–272.

Heinz, E., 1993. Biosynthesis of polyunsaturated fatty acids. In: Moore Jr., T.S. (Ed.), Lipid Metabolism in Plants. CRC Press, Boca Raton, pp. 33–89.

Hilditch, T.P., 1956. The Chemical Constitution of Natural Fats. Chapman & Hall, London.

Ichihara, K., Asahi, T., Fujii, S., 1987. 1-Acyl-*sn*-glycerol-3-phosphate acyltransferase in maturing safflower seeds and its contribution to the non-random fatty acid distribution of triacylglycerol. Eur. J. Biochem. 167, 339–347.

Ichihara, K., Noda, M., 1982. Some properties of diacylglycerol acyltransferase in a particulate fraction from maturing safflower seeds. Phytochemistry 21, 1895–1901.

Ichihara, K., Shibahara, A., Yamamoto, K., Nakayama, T., 1996. An improved method for rapid analysis of the fatty acids of glycerolipids. Lipids 31, 535–539 (Erratum 889).

Lands, W.E.M., 1993. Eicosanoids and health. Ann. NY Acad. Sci. 676, 46–59.

Leonard, E.C., 1999. Dietary fatty acids: effect of the *n*-6/*n*-3 balance on human nutrition and disease. Lipid Technol. 11, 110–114.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mattson, F.H., Volpenhein, R.A., 1961. The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides. J. Lipid Res. 2, 58–62.

Noda, M., Obata, T., 1975. Fatty acid distribution in perilla seed lipids. J. Agr. Chem. Soc. Japan 49, 251–256 (in Japanese)

Slack, C.R., Campbell, L.C., Browse, J.A., Roughan, P.G., 1983. Some evidence for the reversibility of the cholinephosphotransferase-catalized reaction in developing linseed cotyledons in vivo. Biochim. Biophys. Acta 754, 10–20.

Slack, C.R., Roughan, P.G., Balasingham, N., 1978. Labelling of glycerolipids in the cotyledons of developing oilseeds by [1-^{14}C]acetate and [2-^{3}H]glycerol. Biochem. J. 170, 421–433.

Slack, C.R., Roughan, P.G., Browse, J.A., Gardiner, S.E., 1985. Some properties of cholinephosphotransferase from developing safflower cotyledons. Biochim. Biophys. Acta 833, 438–448.

Stobart, K., Mancha, M., Lenman, M., Dahlqvist, A., Stymne, S., 1997. Triacylglycerols are synthesised and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L.) seeds. Planta 203, 58–66.

Stobart, A.K., Stymne, S., 1985. The interconversion of diacylglycerol and phosphatidylcholine during triacylglycerol production in microsomal preparations of developing cotyledons of safflower (*Carthamus tinctorius* L.). Biochem. J. 232, 217–221.

Stymne, S., Appelqvist, L.-A., 1978. The biosynthesis of linoleate from oleoyl-CoA via oleoyl-phosphatidylcholine in microsomes of developing safflower seeds. Eur. J. Biochem. 90, 223–229.

Stymne, S., Stobart, A.K., 1984. Evidence for the reversibility of the acyl-CoA:lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons and rat liver. Biochem. J. 223, 306–314.

Stymne, S., Tonnet, L.M., Green, A.L., 1992. Biosynthesis of linolenate in developing embryos and cell-free preparations of high-linolenate linseed (*Linum usitatissimum*) and low-linolenate mutants. Arch. Biochem. Biophys. 294, 557–563.

Tumaney, A.W., Shekar, S., Rajasekharan, R., 2001. Identification, purification, and characterization of monoacylglycerol acyltransferase from developing peanut cotyledons. J. Biol. Chem. 276, 10847–10852.