1-14C Linoleoyl-CoA Desaturation into Diverse Lipid Classes of Dracocephalum moldavica Cotyledons

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Abstract: To elucidate factors that contribute to the high 18:3 content of Dracocephalum moldavica (D. moldavica), a pulse-chase study of radiolabeled 14C linoleoyl-CoA was carried out at an early and late stage of embryo triglyceride (TG) synthesis. In D. moldavica 14C-18:2 incorporation in PC increased and 14C-18:3 decreased, meanwhile the opposite was observed in TG (the incorporation of 14C-18:3 in TG is two-fold that of 14C-18:2 in PC). This suggests that both ω-3 desaturase is effectively desaturating 14C-18:2 in PC and acyltransferases are selectively transferring 14C-18:3 into TG. Also 14C-18:3 increased in D. moldavica TG indicates that most of the 18:2 was desaturated to 18:3 before transfer to the free fatty acid/acyl-CoA pool in D. moldavica.

Keywords: Dragonhead, Lipid classes, Fatty acids.

1. Introduction

Triacylglycerols (TAGs) are the major lipid class in oilseeds. Many plant oils are rich in unsaturated fatty acids incorporated into their TAG. Therefore, the understanding of TAG formation is very important scientific issue (Saraçoğlu et al., 2012). Both desaturation and TAG synthesis take place in the endoplasmic reticulum (ER). Fatty acids synthesis starts in the plastids and the resulting FA are delivered to the cytosol as thioesters derivatives (fatty acyl-CoA pool) (Browse and Somerville, 1991; Ohlrogge et al., 1991; Topfer et al., 1995). Different fatty acyl-CoAs are deposited into membrane and storage lipids in the ER by different types of acyltransferases. ER desaturases modify the fatty acids esterified to lipid membranes specially phosphatidylcholine (PC) (and possibly other phospholipids) (Ohlrogge and Browse, 1995; Weselake, 2000). The fatty acid distribution in TAGs is widely varied, and it is species and variety specific (Roughan and Slack, 1982). In flax seeds, incorporation of 14C from acetate into lipids was very rapid and with phospholipids and 1, 2-diacylglycerols it was within as short as 5 min. Triacylglycerols were labeled much slower (Dybing and Craig, 1968). In the biosynthesis of triacylglycerol the acyl groups are esterified at the sn-1, 2, and 3 positions of the glycerol back bone by the activities of the acyl-CoA: sn-glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA:1-acyl-sn-glycerol-3-phosphate acyltransferase (APAT), and acyl-CoA:1,2 diacylglycerol acyltransferase (DGAT), respectively (Roughan and Slack, 1982; Frentzen, 1986; Frentzen, 1998). Moreover, the fatty acid composition in position 2 of the glycerol back bone can be modified through the reversible acyl-CoA: lysophosphatidylcholine acyltransferase reaction (Stymne and Stobart, 1984), and CDP-choline: diacylglycerol choline phosphotransferase reaction (Ichihara, 1984; Slack et al., 1985). As reviewed by Bernerth and Frentzen (1990) the properties of some different acyltransferases, such as DGAT from Tropaeolum majus and Limnanthes douglaii, have already investigated especially for their fatty acid specificities and selectivity and they fund to be highly specific toward their substrates and this wieldy vary from plant to plant. The synthesis of C18 polyunsaturated fatty acids from oleic acid and their incorporation into TAGs in oilseeds are better understood (Stymne and Stobart, 1987). It has been shown for many species that diacylglycerol (DG) are the precursors of phosphatidylcholine (PC) and TAG. During the formation of triacylglycerols through glycerol-3-phosphate or the Kennedy pathway, (Kennedy, 1961), there is an equilibrium between the DAG pool, PC pool, and glycerol backbone (Slack et al., 1985; Stobart and Stymne, 1985).

Linoleote incorporated into TAG from the acyl-CoA pool occurs through an acyl exchange between oleoyl-CoA and linoleate at position sn-2 of PC. Then oleoyl position in the sn-2 position of PC has been fund to be important in regulating the level of polyunsaturated fatty acids availability for TAG formation. Also in castor, DAG acyltransferase (DGAT) is highly selective for DAG species and utilizes both medium and long-chain acyl-CoA species (Griffiths et al., 1988; Bafor et al., 1990; Lin et al., 1998). In addition, the conversion of PC to free fatty acids by phospholipase A2 is strongly dependent on the enzyme itself and different phospholipases have strong specificity toward specific fatty acids (Lin et al., 1998).
In addition, other acyltransferases such as glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) from different organisms have different specificity toward different fatty acids (Lohden and Frenzen, 1992; Pillai et al., 1998; Sharma et al., 2012). The regulation and the metabolic factors of different oil accumulation levels and their wide range of fatty acid contents in oilseeds are currently unknown (Ohlrogge and Jaworski, 1997). However, sufficient acyltransferase activities and glycerol-3-phosphate levels are not the only limiting factors in TAG synthesis rates in vivo, but the amount of fatty acid produced in plastids may in part determine the amount of TAG synthesized in oilseeds (Bao and Ohlrogge, 1999).

Linoleoyl phosphatidylethanolamine is the main substrate for microsomal \( \omega \)-3 desaturase, but the linoleic acid ester with galactolipids or sulfolipids is the main substrate for plastid isoforms (Kinney, 1994). The linolenate is synthesized much less at the \( sn-1 \) position of PC than that synthesized at the \( sn-2 \) position. In addition there are two genes that control linoleoyl-PC desaturation during the early stages of the high accumulation of TAG in linseed (Stymne et al., 1992). It is now evident that humans need to increase their dietary \( \omega-3 \) fatty acid levels. Oils high in \( \omega-3 \) fatty acids are also more useful renewable resources such as drying oils used in products such as printing inks. So in order to be able to engineer seeds oil to produce such nutritionally and industrially valuable compounds in a relatively lower commercial production expense, it is important to understand the factors that affect high \( \omega-3 \) accumulation in TAG in high producer plants.

Dragonhead is the common name for \( Dracocephalum moldavica \), contains 25-30% oil and 59-68% of it is 18:3 (Hagemann et al., 1967; Domokos et al., 1994; Matthias, 1997; Abdel-Reheem et al, 2003 ). Pulse chase studies were carried out to elucidate the metabolic factors that contribute to the high \( \omega-3 \) content of \( D. moldavica \).

2. Material and Methods

2.1. Plant material

Seeds of two different cotyledons developmental stages; were TG is mostly synthesized; from \( D. moldavica \) (0.05- 0.1 mg d/w& 0.1-0.2 mg d/w), were taken, and seed coats were removed and cotyledons were separated.

2.2. Pulse-Chase

Radiolabeled 18:2 CoA (0.0033 \( \mu \)Ci) / mg fresh seed tissue, 1 \( \mu \)L/mg fresh weight of embryo of 0.1 M potassium phosphate buffer, pH 7.2, 0.1% Tween 20, each time with 1 mL. Two replicates, from each stage of \( D. moldavica \) were taken for lipid analysis (zero time), and the rest were incubated for further chasing. Samples were collected for lipid analysis after 30, 60 (1h), and 120min. (2h), from zero time.

2.3. Separation of lipid classes using TLC

Total lipids were extracted in chloroform: methanol (2:1 v/v). To analyze the incorporation of radiation in different lipid classes, individual lipids were separated by one-dimensional thin layer chromatography 20x20 cm TLC plate (LK60 silica gel 60 Å, Whatman) by the method of (Kumar, 1983). TLC plates were developed in two solvent systems. Phospholipids and glycerolipids were separate in chloroform: methanol: water (65:25:4, v/v) for 10 cm ascending. Neutral lipids were separated in hexane: diethyl ether: acetic acid (100: 100: 2, v/v) for 19.5 cm ascending (Miquel and Browse, 1992). Lipids were located by spraying the plates with solution of 0.005% pramilin in 80% acetone, followed by visualization under UV light. The silica gel from each lipid band were scrapped, transferred to a tube containing 2 mL of 2% (V/V) \( H_2SO_4 \) in methanol, and fatty acid methyl esters were prepared as described above.

2.4. Reverse phase TLC

To separate linoleate (18:2) and linolenate (18:3) a reverse phase TLC approach was applied. Fatty acids methyl esters, for each lipid classes, obtained from the previous steps were loaded into TLC plates (KC18 silica gel 60 Å, Whatman) plates were developed in acetonitrile: acetic acid: water (70: 10: 10 v/v) for 19.5 cm ascending. This plate was stained with iodine. 18:2 and 18:3 bands were scrapped; activity was counted using a liquid scintillation analyzer (Packard, 15000 Tri-Crab).

3. Results & discussions

3.1. \( ^{14}C \) incorporation into different lipid fractions of \( D. moldavica \)

Figure 1 shows the incorporation of \( ^{14}C \) in different lipid fractions especially in 18:2 and 18:3, Figure 1A shows that the activity of \( ^{14}C \)-18:2 increased during the chasing time in PC of the second stage seeds. The same data shows that label in \( ^{14}C \)-18:3 decreased rapidly in the seeds of the same cotyledon developmental stage. However, in the first stage \( ^{14}C \)-18:2 incorporation was almost steady during the different chase times at low level, on the other hand \( ^{14}C \)-18:3 activity decreased after 1h then started to increase after 1h of chasing time. In Figure 1B, \( ^{14}C \)-18:3 activity level in triacylglycerol dramatically increased in the seeds of the second stage during the chase time and became 20 Becquerel/mg seed at 2h, also in the seeds of the first stage it showed a slightly increment after 1/2h of the chase time. On the other
hand the $^{14}$C -18:2 activity level in TG slightly decreased in the first cotyledon developmental stage, while it slightly increased at 1h then decreased at 2h of the chase time which suggests that DGAT activity is very low in this stage of seed development.

The data from these two Figures highly suggests that 18:2 is desaturated into 18:3 with $\omega$-3-desaturase at the PC level (Kinney, 1994) and then 18:3 is rapidly transferred from PC to TG by acyltransferases (Griffiths et al., 1988; Bafor et al., 1990; Lin et al., 1998). 18:2 incorporation into PC during the late chase times is only at low levels due the availability of the $^{14}$C -18:2 fed, meanwhile $^{14}$C -18:3 increased during the chasing times at very high levels which also support the rapid desaturation on PC and transfer into TAG for both 18:2 and 18:3 respectively, (Figures1 A & B). Figure 1C showed that both $^{14}$C -18:3 and $^{14}$C -18:2 levels at a lower scale. However, $^{14}$C -18:3 level decreased after 1/2h chase time, which is supporting the previous conclusion about that acyltransferase is an important key factor in incorporation of 18:3 into TG. This data showed that seeds of the first stage have relatively very low level of both $^{14}$C -18:2 and $^{14}$C -18:3.

In addition Figure 1C showed that the $^{14}$C -18:3 level is higher than $^{14}$C -18:2 at 0h& 1/2h during chasing of the radiation in the seeds of the second stage of development cotyledons, after that $^{14}$C -18:3 slightly decreased and the $^{14}$C -18:2 level increased. The incorporation of $^{14}$C -18:2 and $^{14}$C -18:3 were found to be at a very low level in MG in both cotyledon developmental stages (Figure 1D) as expected. However the same data in Figure 1D shows that both $^{14}$C -18:2 and $^{14}$C -18:3 rapidly decrease after 1/2h in D. moldavica seeds of the second stage emphasizing MG is apparently used by further steps of lipid metabolism e.g. the synthesis of LPA, PA, DG, PC, and finally TG. The formation of radiolabelled MG raises the question about the possibility of having non-glycerol-3-phosphate pathway using diacylglycerol transacylase (DGTA) for TG production from two DG molecules (Lehner and Kuksis, 1993; Stobart et al., 1997) in D. moldavica or from the direct acylation of AGAT as in animal system (Lee et al., 2001). However MG with $^{14}$C-fatty acids level was very low which in turn suggests, that the possibility of having such non-glycerol-3-phosphate routes for TG production is very small and the main route for that is the old glycerol-3-phosphate pathway, or its conversion is too efficient to be accumulated in D. moldavica and then the possibility of having non-glycerol-3-phosphate routes is standing (Abdel-Reheem et al, 2003).

From the data in Figure 1E the level of $^{14}$C -18:2 decreased in free fatty acids fraction in seeds of the second cotyledon developmental stage, also $^{14}$C -18:3 increased until 1/2h and then started to decrease toward the end of the chase time, which emphasizes the important role of other acyltransferases, as well as phospholipases in the incorporation of 18:3 into TG in developing D. moldavica seeds and in the incorporation of the $^{14}$C -18:2 from free fatty acyl-CoA pool into DAG which is used up rapidly to produce $^{14}$C -18:3 through the desaturation of $^{14}$C -18:2 PC and then $^{14}$C -18:3 is transferred from PC into TG the final oil storage form by the functions of both PL and DGAT (Löhrden and Frentzen, 1992; Pillai et al., 1998; Lin et al., 1998). Moreover data showed accumulation in $^{14}$C -18:3 until 1/2h (from the chasing time) which drops fast from 1 to 0.3 Becquerel/mg seed after 1h and 2h of the chasing meanwhile it decreases in PC and accumulated in TG as mentioned above (Figures 1A&1B). Also this suggests that acyltransferases are very active since the level of $^{14}$C -18:3 in the free fatty acid pool during the chase times (Figure 1E) is relatively lower than its level in TG during the same chase times (Figure 1B).

The % of $^{14}$C incorporation into different lipid fractions in D. moldavica cotyledon at two different developmental stages differed (Figure 2). In Figure 2A % of $^{14}$C incorporation in PC at 0h and 1/2h is higher than in TG at the same chasing times. However $^{14}$C incorporation in PC declined to a steady state at 2h, meanwhile $^{14}$C incorporation in TG increased to steady state at 2h. In Figure 2B the $^{14}$C % in TG is higher than that of PC in the second stage. $^{14}$C % in PC slightly increased after 1/2h of chasing and reached a steady state at 1-2h of chasing time. All other lipid fractions have very low $^{14}$C percentage at both first and second stages (Figures 2A& 2B).

This data is in accord with the data from Figure 1, suggesting that the $\omega$-3-desaturase and acyltransferases as well as possibly phospholipases are playing an important role in 18:3 accumulations in D. moldavica seeds and the main route for this is as illustrated in Figure 3, which indicates that 18:2 in PC is desaturated to 18:3 efficiently then rapidly transferred into TG by acyltransferases since PC meanly is a major component of cell membranes, it is the major substrate for 18:1 and 18:2 membrane bound desaturases, and in many oilseeds PC is a direct precursor of the polyunsaturated species of DAG which appears to be the main substrate for TAG synthesis (Browse and Slack, 1981; Slack et al., 1985; Stymne et al., 1992; Thomaeus et al., 2001; Suh et al., 2002; Suryadevara et al, 2008). DAG acyltransferase (DGAT) is highly selective for DAG species as well as acyl-CoA species (Griffiths et al., 1988; Bafor et al., 1990; Lin et al., 1998), and in D. moldavica it seems like it has higher efficiency working on transferring 18:3 over 18:2 into TG, and that the lipid metabolism is optimally adjusted to 18:3 production and incorporation in the storage lipid.
Figure 1(A): $^{14}$C18:2 Incorporation in *D. moldavica* PC

Figure 1(B): $^{14}$C-18:2 incorporation in *D. moldavica* TAG
Figure 1(C): $^{14}$C-18:2 incorporation in *D. moldavica* DAG

Figure 1(D): $^{14}$C18:2 Incorporation in *D. moldavica* MAG
Figure 1: D. moldavica seeds from two different cotyledons developmental stages were fed $^{14}$C-18:2 CoA for 90 minutes, the incorporation of $^{14}$C-18:2 and conversion to 18:3 were chased in the different lipids fractions at 0h, 1/2h, 1h, and 2h. (A) $^{14}$C incorporation in phosphatidyl choline, (B) $^{14}$C incorporation in triacylglycerol, (C) $^{14}$C incorporation in diacylglycerol, (D) $^{14}$C incorporation in free fatty acids, and (E) $^{14}$C incorporation in monoacylglycerol.

Figure 2 (A): % $^{14}$C-18:2 incorporation in lipid fractions in D. moldavica first stage
Figure 2(B): % $^{14}$C-18:2 incorporation in lipid fractions in D. moldavica second stage

Figure 2: The percentage of $^{14}$C incorporation in lipid fractions in D. moldavica cotyledons at two different developmental stages, (A) First cotyledon developmental stage, (B) Second cotyledon developmental stage. Both stages were fed $^{14}$C-18:2 CoA for 90 minutes, the incorporation of $^{14}$C into both 18:2 and 18:3 were chased in the different lipids fractions at 0h, 1/2h, 1h, and 2h.

Figure 3: Suggested illustration for TG synthesis in D. moldavica seeds. As $^{14}$C-18:2 incorporation and its conversion into $^{14}$C-18:3 were chased in different lipid classes; PC, TG, DG, MG, and FFA; in D. moldavica developing seeds. 

This Figure is based on Kennedy pathway (Kennedy, 1961)
In conclusion, D. moldavica $^{14}$C-18:2 incorporation in PC increased and $^{14}$C-18:3 decreased, meanwhile the opposite was observed in TG (the incorporation of $^{14}$C-18:3 in TG is two-fold that of $^{14}$C-18:2 in PC). This suggests that both ω-3 desaturase is effectively desaturating $^{14}$C-18:2 in PC and acyltransferases are transferring $^{14}$C-18:3 into TG. Also $^{14}$C-18:3 increased in D. moldavica TG. Most of the 18:2 was desaturated to 18:3 before transfer to the free fatty acid/acyl-CoA pool in D. moldavica. In addition the pulse chase data for D. moldavica suggest that linoleoyl-PC derived from DG via Kennedy pathway (Kennedy, 1961); is converted to linolenoyl-PC through desaturation by ω-3 desaturase and is converted to DG and subsequently the linolenoyl moieties are rapidly transferred to DG forming TG (Figures 1 A, B and C).

Also from Figures 1 A and B, the rapid decreasing in $^{14}$C-18:3 levels in PC and its increasing in TG gives the impression that D. moldavica DGAT has high efficiency toward the 18:3 incorporation into TG. The high levels of $^{14}$C-18:3 in TG (Figure 1B) suggest that D. moldavica ω-3 desaturase has high efficiency desaturating 18:2 and converting it into 18:3 (Kumar, 1983).

However this does depends on what stage of seed development is used (Privett et al., 1973; Suh et al., 2002). Pulse chase data emphasize the role of acyltransferases in TG and could roll out that more than one pathway may exist for storage lipid biosynthesis in plants (Zou et al., 1997; Slabas et al., 2001). However ω-3 desaturase may have a major role in linolenate accumulation in D. moldavica.

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