Triacylglycerol biosynthesis and gene expression in microspore-derived cell suspension cultures of oilseed rape

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Abstract
The effect of sucrose concentration on triacylglycerol biosynthesis and associated gene expression was examined in a microspore-derived cell suspension culture of oilseed rape (Brassica napus L. cv. Jet Neuf). The triacylglycerol content of the cells increased about 5-fold on a fresh weight basis when the sucrose concentration in the growth medium was raised from 2% to 22% (w/v). The specific activity of microsomal diacylglycerol acyltransferase (EC 2.3.1.20) and its activity per unit fresh weight increased about 2.5-fold and 6-fold, respectively, when sucrose concentration was increased from 2% to 14%. mRNA encoding the major oleosin also appeared to increase in abundance over the 2–14% sucrose concentration range when RNA fractions were analysed by the reverse transcription-polymerase chain reaction. The sucrose-mediated increases in diacylglycerol acyltransferase activity and oleosin mRNA indicated that the cell suspension could be a useful research tool for the identification of cDNAs encoding triacylglycerol biosynthetic enzymes and associated proteins.

Key words: Triacylglycerol, Brassica, sucrose, acyltransferase, differential display.

Introduction
A thorough understanding of the physiological and genetic regulation of triacylglycerol (TAG) biosynthesis in developing seeds of oilseed rape is fundamental to the rational development of biotechnological strategies for increasing seed oil content and manipulating oil composition. In oilseeds, the biosynthesis of TAG in developing seeds is catalysed by the sequential addition of fatty acyl groups to a glycerol backbone derived from sn-glycerol 3-phosphate (Stymne and Stobart, 1990). Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyses the acylation of sn-1,2-diacylglycerol to yield TAG (Stymne and Stobart, 1987). In developing seeds of Brassica napus L., maximum DGAT activity occurs during the rapid phase of oil accumulation (Tzen et al., 1993; Weselake et al., 1993a). The enzyme may also exert significant flux control in TAG bioassembly (Perry and Harwood, 1993a, b). The enzymes of TAG bioassembly are associated with the endoplasmic reticulum (ER) (Stobart et al., 1986). The apparently hydrophobic and labile nature of these enzymes appears to have impeded progress on their purification and characterization (Gibson et al., 1994; Little et al., 1994; Eccleston and Harwood, 1995; Kocsis et al., 1996). TAG accumulates in developing seeds of B. napus as small droplets, termed oil bodies, which are encapsulated by a single layer of phospholipid (Murphy, 1990; Huang, 1992; Napier et al., 1996). Oleosin is a protein which associates with these droplets (Huang, 1992). There is controversy on whether oleosin synthesis occurs in parallel with TAG synthesis in the ER or whether oleosin and phospholipid are incorporated into lipid bodies following production of ‘naked’ oil droplets.
on the ER (Murphy et al., 1989; Murphy, 1990; Huang, 1992; Napier et al., 1996). Thus, DGAT activity and oleosin accumulation appear to be useful indicators of TAG accumulation in *B. napus*.

Recently, TAG biosynthetic enzymes have been investigated in microspore-derived (MD) cultures of *B. napus* L. (Weselake et al., 1993a, b; Little et al., 1994; Taylor and Weber, 1994; Kocsis et al., 1996). A cell suspension culture of *B. naphthi* L. cv. Jet Neuf was generated in 1983 (Simmonds et al., 1991) and was initially used for studies of freezing tolerance (Orr et al., 1986; Johnson-Flanagan and Singh, 1987; Johnson-Flanagan et al., 1991). The current study examines the effect of sucrose concentration on TAG biosynthesis and gene expression in the MD cell suspension culture of oilseed rape. The effect of sucrose concentration on cell growth, lipid content, fatty acid composition, DGAT activity and relative abundance of oleosin mRNA were determined. Results indicated that the cell suspension system may be useful for identifying genes encoding lipogenic enzymes.

**Materials and methods**

**Chemicals**

[1-14C]Oleic acid (54 Ci mol−1) was obtained from Amersham Canada Ltd., Oakville, Ontario. Acyl-CoAs were synthesized from radiolabelled fatty acids according to Taylor et al. (1990). Silica Gel H was from E Merck, Darmstadt, Germany. HPTLC-Fertigplatten Kieselgel 60 Plates were from the Mandel Scientific Co., Mississauga, Ontario. EcoliteTM (+) biodegradable scintillant was from ICN Biomedicals, Inc., Irvine, California. Dye reagent concentrate for protein assays was from Bio-Rad, Richmond, California. HPLC-grade solvents were from BDH, Inc., Toronto, Ontario. Methanolic-HCl reagent was from Supelco, Inc., Bellefonte, Pennsylvania. sn-1,2-Diolein (15% sn-1,3-diolein) and standard fatty acid methyl esters (FAMES) were from NuChek Prep Inc., Elysian, Minnesota. TRIZOL™ Reagent and other molecular biology biochemicals and reagents were from Life Technologies, Gibco BRL, Gaithersburg, Maryland.

**Plant material**

The MD cell suspension culture of winter oilseed rape (*B. napus* L. cv. Jet Neuf) was provided by Dr J Singh of the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa and maintained according to Orr et al. (1986). Cells were collected, washed with water over a nylon sieve, blotted with filter paper and the fresh weight determined. Cells were used either immediately or frozen in liquid N2, sieved, blotted with filter paper and the fresh weight determined. The grinding medium was 0.2 M HEPES-NaOH buffer (pH 7.4) containing 0.5 M sucrose. The homogenate was filtered through a 60 μm nylon mesh and centrifuged at 10 000 g for 20 min. The resulting supernatant was centrifuged at 100 000 g for 1 h to obtain a microsomal fraction (10 000–100 000 g sediment). Microsomes were washed once and resuspended with grinding medium to a volume equal to one-tenth of the tissue weight. Microsomal DGAT was assayed at 30 °C for 10 min as described by Little et al. (1994) using 60 μl reaction mixture containing 0.2 M HEPES-NaOH (pH 7.4), 330 μM sn-1,2-diolein, 15 μM [1-14C]oleoyl-CoA (54 Ci mol−1), 0.1% (w/v) Tween-20, 5 mg BSA ml−1 and 5–10 μl resuspended microsomes. The protein content of microsomes was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, using BSA as a standard.

**RNA isolation and analysis**

Total RNA was prepared from cells using TRIZOL™ Reagent according to the manufacturer’s instructions. The one-step RNA extraction procedure was a modification of the method described by Chomczynski and Sacchi (1987). Contaminating DNA was digested by treatment with DNase I (Li and Pardee, 1994). The RNA was then treated with phenol/ chloroform and precipitated with ethanol. The RNA pellet was dried and resuspended in water, treated with diethylpyrocarbonate and the concentration of the nucleic acid was determined spectrophotometrically at 260 nm.

**Reverse transcription (RT)-polymerase chain reaction (PCR) of mRNAs encoding oleosin and mitochondrial elongation factor Tu**

mRNA contained in the total RNA fraction was reverse transcribed using the SuperScript II RT enzyme with oligo (dT)15 primer according to the supplier’s instructions (Gibco BRL). RT efficiency was monitored by incorporation of [α-32P]dCTP. The first strand cDNA was amplified by PCR using primers specific to the major oleosin (nap-II) from oilseed rape (*GeneBank Accession X58000*) (Murphy et al., 1991) and the nuclear-encoded mitochondrial elongation factor Tu (*GeneBank Accession X89227*). Primers for amplification of oleosin cDNA were 5′-GACGACTCTGTATTGACGCG-3′ (primer 1) and 5′-ATTCTAAACACCTTATTGC-3′ (primer 2), respectively. Primers for amplification of mitochondrial elongation factor were...
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Tu cDNA were 5'-GTAGGACTTCTTCTGCGTGG-3' (primer 1) and 5'-AACTGTTCCTACCTCCTCC-3' (primer 2), respectively. PCR was conducted with 20–35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C followed by a final 10 min at 72 °C. After amplification of non-radiolabelled cDNA, 10 μl of the reaction mixture was subjected to agarose gel electrophoresis followed by staining with ethidium bromide. Amplified cDNA (579 bp) partially encoding the major oleosin was resolved in 1% agarose + 1% Metaphor agarose. Amplified cDNA (329 bp) partially encoding mitochondrial elongation factor Tu was resolved in 2% agarose + 1% Metaphor agarose.

Results

Effect of sucrose concentration on cell growth, TAG accumulation and fatty acid composition of TL and TAG

The effect of sucrose concentration on the growth of the MD cell suspension cultures of winter oilseed rape (B. napus L. cv. Jet Neuf) was investigated. One gram portions of cells were allowed to grow at various sucrose concentrations ranging from 2% to 22% (w/v) for 2 weeks. Cell growth was examined by measuring the fresh weight (FW) and dry weight (DW) following growth at different sucrose concentrations. The FWs of the cells were highest in media with 2% to 6% sucrose (Fig. 1A). The FW, however, declined about 2-fold between 6% and 10% sucrose. Maximum DW of recovered cells was obtained in 6% sucrose (Fig. 1B). Cell morphology as assessed by scanning electron microscopy, after 2 weeks of growth, did not appear to be affected by alterations in sucrose concentration (not shown). Numerical relationships between FW, DW, TL and TAG are presented in Table 1. Per cent DW increased more than 2-fold as the sucrose concentration increased from 2% to 14%. On a FW basis, TAG content increased more than 5-fold in cells cultured in 22% sucrose over those grown in 2% sucrose while the increase was 2-fold when results were expressed on a DW basis. There was an enrichment in the proportion of TAG in TL at sucrose concentrations above 2%. The fatty acid composition of TL and TAG in the cells cultured at different sucrose concentrations is shown in Table 2. In all cases 18:1 was the most abundant fatty acid. The 18:1 was a mixture of cis Δ-7 (vaccenic) and cis Δ-9 (oleic) forms which were poorly resolved with the chromatographic conditions used. The very long chain saturated fatty acids (20:0, 22:0 and 24:0) accounted for about 23% and 9% of the fatty acids in TL and TAG, respectively, when cells were grown in 2% sucrose. The abundance of these fatty acids, however, was reduced in TL and TAG as the sucrose concentration of the growth medium increased. Culturing in 22% sucrose resulted in a 4-fold and 2-fold decrease, respectively, in the abundance of these very long chain fatty acids in TL and TAG when compared to the lipids from cells grown in 2% sucrose.

Effect of sucrose concentration on induction of DGAT activity

The increase in TAG content of cells grown at higher sucrose concentrations suggested an elevation in the activity of TAG biosynthetic enzymes. Therefore, in another series of experiments, DGAT activity was measured in microsomes prepared from cells that had been cultured in 2, 6 and 14% (w/v) sucrose (Fig. 2). The specific activity of DGAT (Fig. 2A) and its total activity g−1 FW of cells (Fig. 2B) increased about 2.5-fold and 6-fold, respectively, between sucrose concentrations of 2% and 14%. The largest increases in DGAT activity, however, were obtained when sucrose concentrations were increased from 2% to 6%.
Isolation of the TAG fraction and subsequent methylation of TL and TAG was performed as described in the Materials and methods. FAMES were determined by GLC and values for TL and TAG were based on the total integrated area of the chromatogram representing each sample. Lipid preparations from three independent cultures were analysed by GLC and the values were averaged.

### Table 1. Effect of sucrose concentration on the DW, TL and TAG content of MD cell suspension cultures

<table>
<thead>
<tr>
<th>Sucrose concentration (w/v)</th>
<th>DW/FW (%)</th>
<th>TL/DW (%)</th>
<th>TAG/TL (%)</th>
<th>TAG/DW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>7.6</td>
<td>10.5</td>
<td>42.5</td>
<td>4.5</td>
</tr>
<tr>
<td>6%</td>
<td>12.8</td>
<td>9.4</td>
<td>67.4</td>
<td>6.3</td>
</tr>
<tr>
<td>10%</td>
<td>14.5</td>
<td>9.6</td>
<td>66.7</td>
<td>6.4</td>
</tr>
<tr>
<td>14%</td>
<td>17.0</td>
<td>8.8</td>
<td>60.0</td>
<td>5.3</td>
</tr>
<tr>
<td>18%</td>
<td>17.7</td>
<td>11.9</td>
<td>63.7</td>
<td>7.6</td>
</tr>
<tr>
<td>22%</td>
<td>18.1</td>
<td>12.7</td>
<td>71.8</td>
<td>9.1</td>
</tr>
</tbody>
</table>

**Effect of sucrose concentration on expression of oleosin mRNA**

The increase in TAG accumulation and induction of DGAT activity observed between 2% and 14% sucrose prompted an analysis of gene expression in the cells. mRNA encoding DGAT, and perhaps other lipogenic enzymes and associated proteins, could possibly increase in abundance over this sucrose concentration range. RT-PCR was used to assess the level of mRNA encoding the major oleosin following culturing of cells in 2, 6 and 14% sucrose. Results of RT-PCR with two RNA preparations, for each concentration of sucrose, indicated that the cells contained oleosin mRNA (Fig. 3A). Oleosin cDNA increased in abundance as a function of sucrose concentration. This suggested that oleosin mRNA also increased in abundance. In contrast, the amount of cDNA encoding the mitochondrial elongation factor Tu appeared to remain constant over the three concentrations of sucrose (Fig. 3B). Thus, the synthesis of elongation factor Tu did not appear to be induced as a function of increasing sucrose concentration in the growth medium.

### Table 2. Effect of sucrose concentration on the fatty acid composition of TL and TAG in MD cell suspension cultures

<table>
<thead>
<tr>
<th>Sucrose concentration (w/v)</th>
<th>Lipid class</th>
<th>Fatty acid composition (Wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>18:0</td>
</tr>
<tr>
<td>2%</td>
<td>TL</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>13.7</td>
</tr>
<tr>
<td>6%</td>
<td>TL</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>13.9</td>
</tr>
<tr>
<td>10%</td>
<td>TL</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>13.6</td>
</tr>
<tr>
<td>14%</td>
<td>TL</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>14.5</td>
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<td>18%</td>
<td>TL</td>
<td>14.0</td>
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<tr>
<td></td>
<td>TAG</td>
<td>14.5</td>
</tr>
<tr>
<td>22%</td>
<td>TL</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>TAG</td>
<td>12.6</td>
</tr>
</tbody>
</table>

*a tr, Trace, <0.5%.*

**Discussion**

The current study has examined the effect of sucrose concentration on TAG accumulation and oleosin transcript levels in oil-forming MD cell suspension cultures of oilseed rape. Zygotic and MD embryos of *B. napus* have been shown to go through a sequence of developmental stages as they accumulate TAG (Pomeroy *et al.*, 1991; Taylor and Weber, 1994), whereas, in the cell suspension system, the cells remain in a non-differentiated state (Orr *et al.*, 1986; Simmonds *et al.*, 1991). Although the cells were non-differentiated, TAG accumulation could be induced by increasing the sucrose concentration of the growth medium. Increased TAG accumulation, however, was associated with a decrease in the growth of cells at concentrations above 6% sucrose. Very long chain saturated fatty acids (>18 carbons), in both TL and TAG, appeared to decrease in prevalence as sucrose concentrations increased. Sucrose concentration and high osmotic potential in growth media have previously been shown to influence the fatty acid composition of plant cultures. For example, increasing sucrose concentrations in the growth media of asexual embryos of *Theobroma cacao* L. cultured *in vitro* resulted in a shift from mostly polyunsaturated fatty acids to saturated and monounsaturated fatty acids in TL (Pence *et al.*, 1981). The growth of the *T. cacao* L. embryos was also substantially inhibited at higher sucrose concentrations with essentially no growth occurring at 21% sucrose. In another study, Finkelstein and Somerville (1989) demonstrated that high osmoticum, due to the addition of 0.69 M sorbitol, resulted in enhanced accumulation of eicosenoic acid (20:1) and erucic acid (22:1) in the TL of cultured zygotic cells.
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Fig. 3. RT-PCR of mRNAs encoding the major oleosin (nap-II) and nuclear-encoded mitochondrial elongation factor Tu using RNA extracted from cells grown in 2, 6 and 14% (w/v) sucrose. (A) Results for independent RT-PCRs of oleosin mRNA for two different preparations of RNA at each sucrose concentration. Primers for amplification of oleosin cDNA were 5’-GACCAGTATTCTATGATCGG-3’ (primer 1) and 5’-AATTCTAAACCTTATTGC-3’ (primer 2). A 579 bp section of the cDNA was amplified. Lanes 1 and 2 are from cells cultured in 2% sucrose, lanes 3 and 4 are from cells cultured in 6% sucrose, and lanes 5 and 6 are from cells cultured in 14% sucrose. Lane 7 represents a 100 bp ladder. (B) Results of RT-PCRs for nuclear-encoded mitochondrial elongation factor Tu transcripts. Primers for amplification of elongation factor Tu were 5’-GTAGGACTTCTTCTGCGTGG-3’ (primer 1) and 5’-AACTGTTCTACCTCCTTCCC-3’ (primer 2). A 329 bp section of the cDNA was amplified. Lanes 1, 2 and 3 represent 2, 6 and 14% sucrose, respectively. Lane 4 represents a 100 bp ladder.

Fig. 2. Effect of sucrose concentration on the (A) specific activity of DGAT and (B) total DGAT activity per unit FW in MD cell suspension cultures. DGAT was assayed in the microsomal fraction (10000–100 000 g). Results are based on averages of four independent cultures.

Embryos of *B. napus* L. cv. Nugget. The basic culture medium contained 0.06 M sucrose (~2%, w/v).

In developing zygotic embryos of *B. napus*, DGAT activity may limit the amount of carbon flowing into TAG (Perry and Harwood, 1993a, b). Furthermore, the activity of DGAT in developing seeds of *B. napus* has been shown to increase markedly during the active phase of oil accumulation and then decrease as seed oil levels reached a plateau (Tzen et al., 1993; Weselake et al., 1993a). In the current study, the increase in TAG accumulation in the cell suspension culture between 2% and 14% sucrose was also accompanied by a large increase in DGAT activity per unit FW. Ross and Murphy (1993) have suggested that increasing sucrose content or the carbon/nitrogen ratio of the growth medium of TAG-forming plant cell cultures may direct more carbon from membrane lipid formation into TAG by induction of DGAT. Induction of TAG accumulation, without associated embryogenesis, suggested that the cell suspension system would be useful for identification of mRNAs encoding lipid biosynthetic enzymes. Many of the genes associated with embryo development or cellular differentiation are apparently not expressed in this system. This should increase the probability of identifying genes associated with TAG biosynthesis. The detection of the oleosin transcript and the apparent upregulation of this transcript as a function of increasing sucrose concentration served as a positive control for one facet of gene expression associated with TAG accumulation in this system. Northern analysis has previously indicated that *B. napus*
oleosin mRNAs are seed specific and accumulate in zygotic and MD embryos (van Rooijen et al., 1992). In studies with TAG-forming cultures of Pimpinella anisum L., Radetzky and Langheinrich (1995) have demonstrated that a close association exists between sucrose supply and TAG content and the abundance of an 18.4 kDa oleosin. Limiting sucrose in the medium resulted in TAG degradation with a concomitant decrease in immunodetectable oleosin.

The effect of different sucrose concentrations in the growth media on both TAG biosynthesis and expression of oleosin mRNA may be linked to both an increase in carbon supply and a change in osmotic potential. The effect of osmoticum on enhancing TAG accumulation has been noted for several tissues, including somatic cultures of Daucus carota L. (Dutta and Appelqvist, 1989; Ross and Murphy, 1993), developing embryos of Triticum aestivum L. (Rodriguez-Sotres and Black, 1994), cultures of Pimpinella anisum L. (Radetzky and Langheinrich, 1994), and cultured somatic embryos of Picea glauca (Moench) Voss (Attree et al., 1992). In general, the effects of osmoticum in promoting increased TAG accumulation were enhanced by the addition of abscisic acid. It has proven difficult, however, to discern the relative contributions of sucrose as a carbon source versus sucrose as an osmoticum in influencing both lipid accumulation and fatty acid composition (Khuri and Moobry, 1995).

In conclusion, the current study has investigated cell growth and lipid content in MD cell suspension cultures of B. napus as a function of sucrose concentration. TAG content, DGAT activity and the abundance of oleosin mRNA all increased when the sucrose concentration of the growth medium was increased from 2% to 14%. Thus, the cell suspension may be useful for identifying genes encoding TAG biosynthetic enzymes. The cell suspension system may also prove useful for investigating both the biochemical and genetic regulation of TAG biosynthesis in oilseed rape in the context of parameters such as increased carbon supply and osmotic potential.

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