Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos

Integration with storage-lipid and storage-protein synthesis and implications for the mechanism of oil-body formation

Denis J. MURPHY*, Ian CUMMINS and Angray S. KANG

Department of Biological Sciences, University of Durham, Science Laboratories, South Road, Durham DH1 3LE, U.K.

The synthesis of the major protein and lipid storage reserves during embryogenesis in oilseed rape (*Brassica napus* L., cv. Mikado) has been examined by biochemical, immunological and immunocytochemical techniques. The mature seeds contained about 45 % (w/w) storage oil and 25 % (w/w) protein. There were three major seed protein components, i.e. about 40–50 % total protein was cruciferin, 20 % was napin and 20 % was a 18 kDa hydrophobic polypeptide associated with the proteinaceous membrane surrounding the storage oil bodies. Embryogenesis was divided into four overlapping stages with regard to the synthesis of these storage components: (1) for the first 3 weeks after flowering, little, if any, synthesis of storage components was observed; (2) storage-oil synthesis began at about week 3, and maximal rates were from weeks 4 to 7; (3) synthesis of the soluble storage proteins cruciferin and napin started at week 6 and rates were maximal between weeks 8 and 11; (4) the final stage was the synthesis of the 19 kDa oil-body polypeptide, which started at weeks 8–10 and was at a maximal rate between weeks 10 and 12. The synthesis of the 19 kDa oil-body protein therefore occurred independently of the synthesis of the soluble seed storage proteins. This former synthesis did not occur until shortly before the insertion of the 19 kDa polypeptide into the oil-body membrane. No evidence was found, either from sucrose-density-gradient-centrifugation experiments or from immunogold-labelling studies, for its prior accumulation in the endoplasmic reticulum. Conventional and immunogold-electron-microscopic studies showed that oil bodies were synthesized in the early to middle stages of seed development without a strongly electron-dense membrane. Such a membrane was only found at later stages of seed development, concomitantly with the synthesis of the 19 kDa protein. It is proposed that, in rapeseed embryos, oil bodies are initially formed with no proteinaceous membrane. Such a membrane is formed later in development after insertion by ribosomes of the hydrophobic 19 kDa polypeptide directly into the oil bodies.

INTRODUCTION

Seed storage reserves are laid down in specific tissues during embryogenesis. In the case of rapeseed (*Brassica napus*), the storage reserves, the vast majority of which are made up of lipid and protein, are almost exclusively localized in the cotyledons of the maturing embryo (Appelqvist, 1972). The ratio of the two major storage products varies between different rapeseed varieties, but, in most of the cultivars in current use, the storage lipid/protein ratio is about 2:1 (w/w). Despite the growing economic importance of rapeseed, and an oft-expressed wish to modulate the quality of its storage lipids and proteins (Podmore, 1986; Robbelen, 1984), relatively little is known about the mechanism and control of the biosynthesis of these storage products.

Seed storage lipids and proteins are localized in organelles termed oil bodies and protein bodies respectively. The lack of knowledge concerning the fundamental mechanisms of oil-body and protein-body formation is reflected in the conflicting views presented in the literature (Wanner & Theimer, 1978; Gurr, 1980; Adams, 1983; Ichihara, 1982; Herman, 1987; Murphy &

Cummins, 1988). This is particularly true for oil-body synthesis, where, until lately, most of the research involved purely structural studies using conventional techniques of electron microscopy (EM). It has recently been reported by several groups that, in many oilseeds, the storage oil bodies are surrounded by a proteinaceous membrane largely made up of a very few polypeptides that are not found elsewhere in the cell (Qu *et al.*, 1986; Herman, 1987; Murphy & Cummins, 1988). Unique oil-body polypeptides have now been purified and used for immunocytochemical studies in maize (*Zea mays*) (Fernandez *et al.*, 1988), rapeseed (Murphy *et al.*, 1988b) and soybean (*Glycine max*) (Herman, 1987). The use of organelle-specific markers, such as antibodies to the various oil-body polypeptides, opens up new possibilities for the investigation of the mechanism of oil-body formation in developing seed tissues.

In the present study, a combined biochemical, immunological and structural approach has been used to investigate the biosynthesis of the major oil-body membrane protein in rapeseed. The relationship between the biosynthesis of the oil-body protein and that of the seed storage oil and the seed storage proteins has also

Abbreviations used: e.l.i.s.a., enzyme-linked immunoadsorbent assay; EM, electron microscopy/scopic; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; PBS and PBST are defined in the text; BSA, bovine serum albumin.

been studied. The results indicate that embryogenesis in rapeseed can be divided into at least four stages with respect to the synthesis of seed storage products, i.e. (i) a lag period, (ii) mainly oil synthesis, (iii) mainly storage protein synthesis and finally (iv) mainly oil-body protein synthesis. The implications of these data for the mechanism of oil-body formation in rapeseed are discussed.

EXPERIMENTAL

Chemicals

All solvents were of AnalaR grade. Biochemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

Plant material

A commercial variety of rapeseed (*Brassica napus* L., cv. Mikado) was used in the present study. Rapeseed was harvested from a field crop near Shincliffe, Co. Durham, from May to July, 1987. Plants were also grown in glasshouses or constant-environment cabinets at the Department of Biological Sciences of this University.

Lipid analysis

Total lipids were extracted from five cotyledon pairs of each age of seedling. Cotyledons were thoroughly homogenized in 20 ml of chloroform/methanol (2:1, v/v), and aqueous contaminants partitioned into the upper (aqueous) phase after the addition of 0.2 vol. of 0.7% KCl. Upper and lower phases were repeatedly washed with pure lower-phase and upper-phase solvents respectively (Folch *et al.*, 1957). The combined lower phases were dried under a stream of N₂ gas and the residual oil was weighed. Alternatively, fatty acid methyl esters were prepared as described previously (Murphy *et al.*, 1983). The methyl esters were fractionated on a Perkin-Elmer 8310 chromatograph using a 2 m column packed with 10% di(ethylene glycol) succinate on Chromosorb W (AW 100–120 mesh), eluted isothermally at 180 °C, and detected by flame ionization. Methyl ester peaks were quantified by reference to a methyl heptadecanoate internal standard.

Protein analysis

Total proteins were assayed in the presence of SDS by a modified Lowry method (Markwell *et al.*, 1981). Protein extracts were solubilized before SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) by incubation at 90 °C for 5 min in 1.7% SDS/1% 2-mercaptoethanol/16% sucrose/0.1 M-Tris/HCl, pH 6.8. Electrophoresis was normally performed using 1.5 mm slab gels containing 15% (w/v) polyacrylamide. Polypeptides were stained with Coomassie Brilliant Blue R. Relative proportions of the mass in each polypeptide were estimated after scanning of the stained gels with an LKB 2222-010 Ultrosan XL laser densitometer.

Isolation of subcellular fractions

All operations were performed at 0–4 °C. Embryos were dissected out from freshly harvested developing seeds. The embryos were gently homogenized using a pestle and mortar. The homogenization medium (4 ml/g of cotyledons) contained 0.4 M-sucrose, 100 mM-Hepes/NaOH, pH 7.5, 10 mM-KCl, 1 mM-MgCl₂ and 1 mM-EDTA (buffer A). The homogenate was filtered through

four layers of cheesecloth and centrifuged at 500 g for 5 min. For the separation of subcellular organelles, the supernatant was layered on a linear 10–50% (w/v)-sucrose density gradient (20 ml) with a 2 ml 70%-sucrose cushion (Lord *et al.*, 1973). All the sucrose solutions contained 1 mM-EDTA at pH 7.5. After centrifugation at 100 000 g for 4 h in a Beckman L-2 65B ultracentrifuge with an SW 27.1 rotor (Beckman Instruments), the floating fat-pad was carefully removed and the gradient separated into 0.5 ml fractions with a density-gradient fractionator (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Marker enzymes were assayed as described previously (Murphy *et al.*, 1989a). Purified oil-body membranes were prepared by gently homogenizing freshly harvested embryos in buffer A as described above. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 5000 g for 15 min. The crude oil-body fraction was recovered from the top of the 5000 g supernatant, dispersed in 5 vol. of buffer A and layered beneath a further 20 vol. of buffer A containing 0.1 M-sucrose. This was then centrifuged at 18 000 g for 15 min, after which the oil-body fraction was again recovered from the top of the gradient. This dispersal-layering-centrifugation procedure was repeated a further three times. The resultant floating oil-body fraction was essentially free of contamination by soluble proteins or other cell membrane components, as judged by SDS/PAGE and marker-enzyme assays (Hills & Murphy, 1988; Murphy *et al.*, 1989a). The purified oil bodies were extracted three times in 5 vol. of diethyl ether in order to remove triacylglycerols. The resulting membranes were pelleted by centrifugation at 100 000 g for 1 h.

Purification of the oil-body and storage proteins

The major oil-body membrane protein was a hydrophobic polypeptide of apparent molecular mass 19 kDa. Membrane proteins were solubilized in a solution containing 40 mg of total oil-body protein/ml and 20 mg of Triton X-100/ml. The 19 kDa protein was then purified to homogeneity after two cycles of SDS/PAGE using 3 mm-thickness gels. Cruciferin and napin were purified by ion-exchange chromatography on DEAE-cellulose DE-52 and gel filtration on Sephadex G-200. The purified proteins were at least 99% pure as judged by SDS/PAGE. In the rapeseed variety used in the present study, cruciferin polypeptides had electrophoretic mobilities equivalent to 20, 22, 29 and 33 kDa and napin polypeptides to 4 and 9 kDa.

Antiserum preparation

Purified proteins were emulsified with complete Freund's adjuvant, and samples equivalent to 100 µg and 500 µg of protein were injected intraperitoneally and intradermally into a mouse and a rabbit respectively. After 4 weeks the animals were boosted with a similar amount of antigen in incomplete Freund's adjuvant. After 2 weeks the animals were bled and serum prepared. Serum from either animal was equally efficient in binding to either the native or denatured forms of the 19 kDa protein. By contrast, anti-napin and anti-cruciferin sera bound preferentially to the native forms of these proteins. The antisera were monospecific to their respective proteins, as judged by immunoblotting of extracts of total cotyledon protein separated by SDS/PAGE (Murphy *et al.*, 1989b).

Immunological assays

The detection of the 19 kDa protein, cruciferin or napin by enzyme-linked immunoadsorbent assay (e.l.i.s.a.) was performed using a modification of the method of Kang *et al.* (1988). Samples were diluted in 0.2 M-Tris/0.3 M-NaCl, pH 9.5, to 1 µg of protein/ml, and 200 µl was loaded to the wells of microtitration plates (Nunc, Immunoplate 1, Gibco) and incubated for 18 h at 4 °C. Plates were washed five times with 10 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7.2)/1.38 mM-NaCl/2.7 mM-KCl (PBS) containing 0.05% Tween 20 (PBST) using a Nunc Immunowash 8 hand-held plate washer and rinsed twice with water. The sample wells were post-coated by incubating 300 µl of PBS containing 1.0% BSA (98–99%) albumin; Sigma) for 1 h at ambient temperature and then washed as described above. Antibodies were diluted to various extents (1:1000–1:50000) in PBST/0.1% BSA, 200 µl was added to the wells and incubated at ambient temperature for 2 h, after which the washing procedure was repeated, followed by the addition of sheep anti-mouse or goat anti-rabbit IgG-horseradish peroxidase conjugate diluted (1:1000) in PBST/0.1% BSA (200 µl/well). After a further incubation of 2 h at ambient temperature the plate was washed and the colorimetric substrate 1.1 mM-azinobis-(3-ethylbenzthiazolinesulphonic acid)/11 mM-citrate buffer (pH 4.0)/

0.071% H_2O_2 was added at 200 µl/well. After an incubation of 30–60 min at ambient temperature, the $A_{415\text{ nm}}$ of each well was determined by using a Titertek Multiscan MCC (Flow Laboratories) microtitration plate reader. Absorbances of the wells were directly proportional to the concentration of the appropriate antigenic protein in the samples.

EM and immunocytochemistry

Tissue pieces (1 mm²) for conventional transmission EM were fixed in 2.5% (w/v) glutaraldehyde/1.5% (v/v) paraformaldehyde/0.05 M-cacodylate buffer, pH 7.0, for 16 h at 4 °C. Post-fixation in 1% OsO_4 was for 6 h. Samples for immunocytochemical studies were fixed in 3% paraformaldehyde/1.25% glutaraldehyde/0.05 M-phosphate buffer, pH 7.0, for 16 h at 4 °C. Samples were then dehydrated through a graded alcohol series followed by infiltration and embedding in Spurr's resin for conventional EM or in LR white resin for immunocytochemistry. Resin was polymerized at 65 °C overnight, LR resin being polymerized in the absence of air. Gold/silver sections were cut using an LKB Ultratome 4801A. For conventional EM, sections were sequentially stained with saturated aqueous uranyl acetate for 15 min and Reynold's lead citrate for 15 min.

Immunogold labelling was performed on ultra-thin sections mounted on to Formvar-coated 200-mesh copper grids. Sections were blocked with 1% BSA in 10 mM-phosphate buffer (pH 7.2)/1.4 mM-NaCl/0.05% Tween 20 (PBST) for 10 min, washed thrice in PBST and incubated for 30 min with anti-(19 kDa polypeptide), anti-napin or anti-cruciferin serum diluted from 1:10 to 1:10000 in PBST. Sections were then washed ten times in PBST and incubated for 30 min with a 1:20 dilution of either sheep anti-mouse or goat anti-rabbit secondary antibody conjugated to 20 nm gold particles. After a further ten washes in PBST, sections were post-stained with aqueous uranyl acetate.

RESULTS

In the present study the biosynthesis of seed storage proteins and lipids was monitored in a commercial variety of winter rape. Most of the data were obtained from field-grown crops growing on farms in North-East England during Spring–Summer, 1987. Cultivation of rapeseed in glasshouses or in the laboratory resulted in a more rapid development and maturation of the seed embryos. Where data were obtained from non-field-grown plants, the developmental stage has been normalized to the equivalent stage in the field crop. The maturation time, from anthesis to dihisence, of field-grown winter rapeseed varied according to many factors, including climate, soil type, drainage and aspect. Typical maturation times were between 10 and 15 weeks.

The change in the polypeptide composition of developing rapeseed embryos is shown in Fig. 1. Protein loadings were made on a per-embryo basis. The absolute amount of protein per embryo increased dramatically during development. The major polypeptides of the seed storage proteins, cruciferin and napin, accumulated throughout embryo development. By contrast, the major oil-body protein, a 19 kDa polypeptide, was present only at the later stages of embryogenesis.

The synthesis of proteins and lipids during embryogenesis is shown quantitatively in Fig. 2. No storage

Fig. 1. Polypeptide composition of developing rapeseed embryos

The ages of the embryos (in weeks) are indicated at the bottom of each lane. Abbreviations: S, molecular-mass standards; M, mature seed; C, cruciferin polypeptides; N, napin polypeptides; O, 19 kDa oil-body polypeptide.

Fig. 2. Synthesis of triacylglycerol, total protein and the 19 kDa oil-body membrane polypeptide during embryogenesis in rapeseed

●, Triacylglycerol; ○, total embryo protein; □, 19 kDa protein (e.l.i.s.a.); △, 19 kDa protein (SDS/PAGE). Triacylglycerols were assayed by g.l.c. or gravimetrically, total embryo protein was assayed by a modified Lowry method, and 19 kDa protein was assayed either by SDS/PAGE-densitometry or by e.l.i.s.a.

Fig. 3. Synthesis of the major seed proteins during embryogenesis in rapeseed

□, Cruciferin; △, napin; ○, 19 kDa oil-body protein. Protein amounts were estimated both by e.l.i.s.a. and by densitometry of SDS/PAGE-separated polypeptides. Both techniques gave very similar results (see Fig. 2), but, for the sake of clarity, only the data from SDS/PAGE densitometry are shown in this Figure.

lipid was evident until after the third week after flowering. Storage triacylglycerols were then formed at a rapid rate until week 7 and at a steady, but somewhat lower, rate thereafter until seed desiccation. Total embryo protein increased at an accelerating rate from week 3 until week 10, and at a lower rate thereafter. Very little trace of the 19 kDa oil-body protein was seen until week 8, and the most rapid phase of its synthesis was between weeks 10 and 12. There was no correlation, therefore, between the synthesis of storage triacylglycerol and the synthesis of the major oil-body protein.

The total embryo protein contained many non-storage-protein components, particularly at early stages of seed development. We wished to determine the individual time courses of the synthesis of the major seed proteins during embryogenesis. There are only three major classes of protein in mature rapeseed, i.e. 40–50% (w/w) is made up of the various polypeptides (20–33 kDa) of the legumin-like neutral storage protein, cruciferin, about 20% (w/w) is made up of the basic protein napin (4–9 kDa) and about 20% (w/w) is made up of the major oil-body protein (19 kDa). None of these proteins has any known catalytic function and hence they cannot be assayed enzymically. For this reason, all three proteins were purified in our laboratories and monospecific antibodies raised to each one. The time course of their synthesis was then monitored either by e.l.i.s.a. or by densitometric analysis of SDS/PAGE-separated poly-

peptides. The results are shown in Fig. 3. It can be seen that napin and cruciferin were synthesized mostly between weeks 6 and 11, whereas the 19 kDa protein did not appreciably accumulate until after week 10. There was, therefore, no apparent coupling between the synthesis of the two soluble storage proteins on one hand and the synthesis of the 19 kDa protein on the other.

We have recently shown, by means of immunogold labelling, that the 19 kDa rapeseed oil-body protein was exclusively associated with oil-body membranes in cotyledons of either developing or germinating rapeseed (Murphy *et al.*, 1989a,b). It was of interest to ascertain whether, during the course of its synthesis, the 19 kDa protein was transported from another subcellular location before being bound to the oil-body membrane. This was studied in two ways, namely by analysing the distribution of 19 kDa protein either in sucrose density gradients or on immunogold-labelled EM sections taken from embryos of different ages. The results of the former experiment are shown in Fig. 4. The absolute amount of 19 kDa protein per cotyledon was quite different at the different ages, as had already been demonstrated in Figs. 2 and 3. It is clear, however, that at each of the ages studied essentially all of the immunogenic 19 kDa polypeptide was associated with the oil-body fraction, most of which was collected from the top of the gradients as fraction 0. There was no indication of the presence of the 19 kDa protein in the light membrane fraction (tubes 12–15), which contained the endoplasmic-reticulum

Fig. 4. Distribution of 19 kDa oil-body membrane protein in fractions of 10–50%-(w/v)-sucrose density gradients after the separation of organelles and membranes from total homogenates of developing rapeseed cotyledons (a) 6 weeks, (b) 9 weeks and (c) 11 weeks after flowering

(a)–(c) ●, 19 kDa protein; (d) ○, CDP-choline:diacylglycerol transferase activity; △, [sucrose].

marker enzyme CDP-choline:diacylglycerol acyltransferase. These data are consistent with the direct insertion of the 19 kDa protein into oil-body either during, or immediately after, its synthesis on ribosomes.

The results of the conventional EM and EM-immunogold labelling studies with various antibodies are shown in Figs. 5–9. Controls with pre-immune serum or BSA resulted in very little or no gold labelling of the sections. The use of anti-napin or anti-cruciferin sera with sections of mature embryos resulted in the specific labelling of protein bodies, whereas no label was associated with the oil bodies (Fig. 5). In contrast, in similar sections incubated with anti-(19 kDa protein) serum at a dilution of 1:1000, there was a specific labelling of the oil-body membranes (Fig. 6). In both cases, very little label was associated with either the oil-body interiors or with other cellular components. As expected, very few gold labels were seen in sections of younger embryos incubated with anti-19 kDa serum. In Fig. 7, where

sections are presented of 7–9-week-old embryos, relatively few gold labels are visible in each micrograph.

The oil bodies appear to be surrounded by ribosomes, which are probably connected by endoplasmic reticulum, although the latter is not always readily visible in these non-OsO₄-treated sections. These ribosomes may be responsible for the synthesis and direct insertion of the 19 kDa protein into the oil-body membrane.

The conventional electron micrographs shown in Figs. 8 and 9 illustrate the change in appearance of the oil-body surface during development. Before synthesis of the 19 kDa protein, the oil bodies do not have a distinct electron-dense boundary layer (Fig. 8). In this electron micrograph, numerous membranous structures can be seen, including mitochondria, endoplasmic reticulum and plastid membranes, but no equivalent osmiophilic membrane can be discerned around the oil bodies. This supports our previous observation that, in rapeseed embryos, newly formed oil bodies appear to arise as naked oil droplets with no protein (but possibly a phospholipid) coat (Murphy & Cummins, 1988). At the 10-week stage or later, when synthesis of the 19 kDa protein is well underway, the oil bodies are surrounded by a heavily stained osmiophilic surface coat (Fig. 9). This surface membrane can also be seen in the immunogold sections of mature embryos shown in Figs. 5 and 6.

DISCUSSION

The results presented here indicate that the synthesis of the major storage reserves during embryogenesis in rapeseed can be divided into four main stages. The first stage was from 0 until 3 weeks after flowering, when little or no accumulation of storage products occurred. The second stage was the synthesis of the storage triacylglycerols. This started after 3 weeks, and the maximum rate was from 4 to 7 weeks, although storage oils continued to accumulate until seed maturity. The third stage was the synthesis of the seed storage proteins, cruciferin and napin. This started after 6 weeks, and maximal rates of storage protein accumulation were found between 8 and 11 weeks. The fourth and final stage was the synthesis of the 19 kDa oil-body protein. This started between 8 and 10 weeks and the maximum rate was from 10 to 12 weeks. Although some of these stages overlap, there is a clear difference in the timing of the formation of the storage lipid, the storage protein and the packaging of the storage lipid by the 19 kDa oil-body protein.

It has been reported recently that oil bodies in young rapeseed embryos were formed as apparently naked oil droplets and that the acquisition of an electron-dense proteinaceous membrane occurred only at the final stages of embryogenesis (Murphy & Cummins, 1988). This observation is confirmed in the present study by the use of a combination of biochemical, conventional EM and EM-immunogold labelling techniques. Our conclusions are consistent with a previous report that a microsomal fraction from developing safflower (*Carthamus tinctorius*) embryos, when supplied with the appropriate precursors for triacylglycerol synthesis, catalysed the formation *in vitro* of naked oil droplets (Stobart *et al.*, 1986). In the light of these and other findings (Adams *et al.*, 1983; Ichihara, 1982; Herman, 1987; Murphy *et al.*, 1989b), it may be necessary to revise

Fig. 5. Immunogold-labelled sections of cells from cotyledons of 12-week-old rapeseed embryos

Sections were indirectly labelled with (a) anti-napin and (b) anti-cruciferin sera, at a 1:10 dilution, followed by a secondary antibody conjugated to 2 nm gold particles. In each case, the gold labels are exclusively associated with the protein bodies (PB), whereas the oil-bodies (O) remain unlabelled. At this developmental stage, the oil bodies were surrounded by a strongly stained proteinaceous membrane, mostly made up of a single type of 19 kDa polypeptide. The bars represent 1 μ m.

Fig. 6. Immunogold-labelled sections of cells from cotyledons of 12-week-old rapeseed embryos

Sections were indirectly labelled with serum raised against the 19 kDa oil-body protein, followed by a secondary antibody conjugated to 20 nm gold particles. Both sections show specific labelling of the oil-body membranes with gold particles. In (a) the anti-19 kDa serum was diluted 1:100, whereas in (b) the anti-19 kDa serum was diluted 1:1000. The bars represent 1 μ m.

our ideas concerning the mechanism of oil-body formation. This has been the subject of some dispute in the past. Several investigators have proposed that naked oil droplets are formed directly in the cytosol of the storage cells (Harwood *et al.*, 1972; Rest & Vaughan, 1972; Smith, 1974; Bergfeld *et al.*, 1978; Gurr, 1980; Ichihara, 1982; Murphy & Cummins, 1988). The most widely accepted hypothesis, however, is that the mature oil bodies are formed by budding-off from the

endoplasmic reticulum (Frey-Wyssling *et al.*, 1983; Schwarzenbach, 1971; Wanner & Theimer, 1978). Despite extensive searching of EM sections of developing embryos from various oilseed species, we were unable to see more than a very few instances of apparent budding-off of oil bodies from the endoplasmic reticulum. Neither was any trace of the characteristic endoplasmic-reticulum polypeptides (Hills & Murphy, 1988) ever found to be associated with oil-body membranes. At present it is not

Fig. 7. Immunogold-labelled sections of cells from cotyledons of 8-week-old rapeseed embryos

Sections were indirectly labelled with serum raised against the 19 kDa oil-body protein, followed by a secondary antibody conjugated to 20 nm gold particles. Both sections show the presence of endoplasmic reticulum (ER) directly appressed against the surface of the oil bodies (OB). At this stage the oil bodies are not surrounded by the electron-dense proteinaceous boundary seen at later developmental stages (e.g. Figs. 5, 6 and 9). The bars represent 0.5 μm .

Fig. 8. Electron micrograph of a section of a cell from a cotyledon of an 8-week-old rapeseed embryo

Numerous oil bodies (O, o) can be seen distributed throughout the cytoplasm. The oil bodies are not surrounded by an osmiophilic boundary such as is seen around membranous structures such as the endoplasmic reticulum (ER), chloroplasts (C), mitochondria (MT) or protein bodies (p). The bar represents 1 μm .

Fig. 9. Electron micrographs of sections of cells from a cotyledon of a 12-week-old rapeseed embryo

In (a) the cells can be seen to be packed with oil bodies (O) surrounding one or more prominent protein bodies (PB). In (b) a magnified view of the oil bodies is shown, in which the strongly osmiophilic oil-body membranes (M) can be readily seen. The bars represent 1 μm .

possible to disprove the 'budding-off' hypothesis of oil-body formation, but our data strongly suggest that oil bodies are initially formed without a protein-rich membrane. These nascent oil bodies may have been secreted from the endoplasmic reticulum and they may have a surrounding phospholipid monolayer, but they are still able to fuse together and hence grow in size. The insertion into the oil body of its characteristic membrane proteins, most of which are made up of a single species of 19 kDa polypeptide, then occurs towards the end of embryo development. Results of recent '*in vivo*' translation studies have demonstrated that the 19 kDa polypeptide is formed *de novo* at this stage of embryogenesis (Murphy *et al.*, 1989b). The oil-body membrane proteins are probably inserted directly into the organelles after synthesis on ribosomes which are closely associated with the oil-body surface (see Fig. 7).

The exact function of the oil-body membrane protein is not yet clear. It may serve to prevent further coalescence of the mature oil bodies. It may be important in packaging the storage oil and to prevent leakage of the oil or damage to other cell contents during the extreme fluctuations in cellular water content which accompany seed desiccation and imbibition. It may also protect the storage oil from attack by hydrolytic enzymes during seed desiccation or during the first few days of germination. Since the oil-body membrane protein is not broken down during the mobilization of the storage protein (Murphy *et al.* 1989a), it must be resistant to digestion by the proteinases which are active at this stage of seed germination. A final possible function is that the oil-body protein may serve as a binding site for the lipase responsible for triacylglycerol breakdown during the mid-phase of seed germination (Huang *et al.*, 1987; Vance & Huang, 1987).

The 19 kDa oil-body membrane protein is a major component of mature rapeseed, comprising 20–25% of total seed protein. We have recently demonstrated that, in germinating rapeseed cotyledons, the 19 kDa oil-body

protein was degraded at the same stage and at the same rate as the storage lipid (Murphy *et al.*, 1989a). In such a tissue, mobilization of the storage protein occurred first and was independent of the mobilization of either the 19 kDa oil-body protein or of the storage oil itself. It appears, therefore, that there is no common regulation of either the synthesis or the breakdown of the soluble seed storage proteins with that of the oil-body membrane protein. Also, although the breakdown of the 19 kDa oil-body protein occurred simultaneously with that of the storage lipid, the synthesis of these two oil-body constituents during embryogenesis was separated by many weeks. This implies that there are several sets of seed-specific genes which are sequentially activated at different times during embryogenesis. It is possible that there exists more than one type of seed-specific regulatory DNA sequence or 'promoter', depending upon at which stage of embryogenesis the gene expression is required. This possibility requires further investigation.

The data presented here suggest that, in the cotyledons of young developing rapeseed embryos, storage oils are synthesized initially as small protein-free droplets which gradually coalesce into larger, but still immature, oil bodies. The storage proteins cruciferin and napin begin to accumulate in mid-development and accumulate in large protein bodies. The final stage in oil-body maturation is the synthesis and insertion of a specific 19 kDa protein into the oil-body membrane.

We thank Dr. A. Ryan for provision of the antibodies to napin and cruciferin. This work was supported by grants from the Agriculture and Food Research Council (U.K.) and the NATO Science Council.

REFERENCES

- Adams, C. A., Norby, S. W., Rinnie, R. W. (1983) *Crop Sci.* **23**, 757–759
- Appelqvist, L. A. (1972) in *Rapeseed* (Appelqvist, L. A. & Ohlson, R., eds.), pp. 123–173, Elsevier, Amsterdam

- Bergfeld, R., Hong, Y. N., Kihnl, T. & Schopfer, P. (1978) *Planta* **143**, 297–307
- Fernandez, D. E., Qu, R., Huang, A. H. C. & Stachelin, L. A. (1988) *Plant Physiol.* **86**, 270–274
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Frey-Wyssling, A., Grieshaber, E. & Muhlethaler, K. (1963) *J. Ultrastruct. Res.* **8**, 506–516
- Gurr, M. I. (1980) in *Biochemistry of Plants* (Stumpf, P. E. & Conn, E. E., eds.), vol. 4, pp. 205–248, Academic Press, New York
- Harwood, J. L., Sodja, A., Stumpf, P. K. & Spurr, A. R. (1972) *Lipids* **6**, 851–854
- Herman, E. M. (1987) *Planta* **172**, 336–345
- Hills, M. J. & Murphy, D. J. (1988) *Biochem. J.* **249**, 687–693
- Huang, A. H. C., Qu, R., Wang, S., Vance, V. B., Cao, Y., Lin, Y. (1987) in *The Metabolism, Structure and Function of Plant Lipids* (Stumpf, P. K., Mudd, J. B. & Nes, W. D., eds.), pp. 239–246, Plenum Press, New York
- Ichihara, K. (1982) *Agric. Biol. Chem.* **46**, 1767–1773
- Kang, A. S., Abbott, S. J. & Harris, N. (1988) in *Manipulating Secondary Metabolism in Culture* (Robbins, R. J. & Rhodes, M. J. C., eds.), Cambridge University Press, in the press
- Lord, J. M., Kagawa, T., Moore, T. S. & Beevers, H. (1973) *J. Cell Biol.* 659–667
- Markwell, M. A. K., Haas, S. K., Tolbert, N. E. & Beiber, L. L. (1981) *Methods Enzymol.* **72**, 296–303
- Murphy, D. J. (1988) *Lipids* **23**, 157–163
- Murphy, D. J. & Cummins, I. (1988) in *Biotechnology for the Fats and Oils Industry* (Applewhite, T. E., ed.), American Oil Chemists' Society, Champaign, in the press
- Murphy, D. J., Mukherjee, K. D. & Latzko, E. T. (1983) *Biochem. J.* **213**, 249–252
- Murphy, D. J., Cummins, I. & Kang, A. S. (1989a) *J. Sci. Food Agric.* **46**, in the press
- Murphy, D. J., Cummins, I. & O'Sullivan, J. N. (1989b) in *The Biological Role of Plant Lipids* (Biacs, P. A., ed.), Akademiai Kiado, Budapest, in the press
- Podmore, J. (1986) *The Future Place for Oilseed Rape in Europe*, pp. 1–15, BASF, Ipswich
- Qu, R., Wang, S., Lin, Y., Vance, V. B. & Huang, A. H. C. (1986) *Biochem. J.* **235**, 57–65
- Rest, J. A. & Vaughan, J. G. (1972) *Planta* **105**, 245–262
- Robbelen, G. (1984) *AOCS Monogr.* **11**, 97–106
- Schwarzenbach, A. M. (1971) *Cytobiologie* **4**, 145–147
- Smith, C. G. (1974) *Planta* **119**, 125–142
- Stobart, A. K., Stymne, S. & Hoglund, S. (1986) *Planta* **169**, 33–37
- Vance, V. B. & Huang, A. H. C. (1987) *J. Biol. Chem.* **262**, 11275–11279
- Wanner, G. & Theimer, R. R. (1978) *Planta* **140**, 163–169

Received 9 May 1988/30 August 1988; accepted 22 September 1988