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Oilbody Proteins in Microspore-Derived Embryos of *Brassica napus*[1]

Hormonal, Osmotic, and Developmental Regulation of Synthesis

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ABSTRACT

A number of treatments were tested for their ability to affect the synthesis of oilbody proteins in microspore-derived embryos of rapeseed (*Brassica napus*). Synthesis of the oilbody proteins was determined by [^{35}S]methionine incorporation *in vivo* and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of washed oilbody fractions. Oilbody proteins of approximately 19, 23, and 32 kilodaltons were found to be prominent. These proteins showed differential patterns of regulation. The 19 and 23 kilodalton proteins (oleosins) were greatly enhanced by treatments with abscisic acid, jasmonic acid, and osmotic stress imposed using sorbitol (12.5%). Synthesis of the 32 kilodalton protein was inhibited by abscisic acid and by sorbitol (12.5%), but unaffected by jasmonates. The strong promotion of synthesis of the 19 and 23 kilodalton oilbody proteins appeared to be specific as they are not seen with gibberellic acid treatment or with a stress such as heat shock. Time course experiments revealed that the abscisic acid stimulation of oleosin synthesis is quite rapid (less than 2 hours), reaching a maximum at 6 to 8 hours. The response of the oleosins to abscisic acid is found in all stages of embryogenesis, with a major increase in synthetic rates even in globular embryos on abscisic acid treatment. This suggests that these proteins may accumulate much earlier in embryogenesis than has previously been believed. The 32 kilodalton oilbody-associated protein appears different from the oleosins in several ways, including its distinct pattern of regulation and its unique property, among the oilbody proteins, of undergoing phosphorylation.

The deposition of storage lipids or triacylglycerides in developing oilseed embryos occurs in oilbodies or oleosomes (4, 11). These oilbodies consist of a lipid droplet surrounded by a half-unit membrane (17) and a number of proteins that may play a structural role (8) or possibly provide a docking site for lipases upon germination (14). Oilbody proteins in developing seeds of corn, soybean, and rapeseed accumulate at relatively high levels (8, 12, 13). In rapeseed (*Brassica napus*), a 19 kD oilbody protein has been reported to accumulate to levels as high as 20% of total cellular protein (8). In most species, there are several oilbody-associated proteins of different molecular masses. The most abundant of these are highly lipophilic proteins, called oleosins (10), with a central hydrophobic core that is highly conserved between diverse species (2, 9, 10, 14). The role of these oleosins is not yet clear, but appears in part to involve stabilization of the oilbody. This is suggested by the unique secondary structure predicted from the complete amino acid sequences of these proteins (10, 14). Recently, Tzen *et al.* (13) showed that in a wide range of species there are two distinct isoforms of oleosins that are immunologically cross-reactive between species, but not within a species. These two isoforms are normally distinguishable by M_r as well as by immunological properties and DNA/protein sequence.

We recently showed that the accumulation of oilbody proteins in *B. napus* was sensitive to ABA (12) and to JA,[2] a natural product of *Brassica* embryos (16). It was desireable to determine the specificity of this regulation and to characterize the developmental sensitivity of oilbody protein accumulation to phytohormones. The interrelationship between osmoticum- and ABA-regulated processes in microspore-derived embryos has also been discussed recently for *Brassica* storage protein genes (15). Therefore, we compared the response of oilbody proteins to applied osmotic stress with that found on ABA treatment. Using microspore-derived embryos of *B. napus* as a source of material representing defined stages of embryogenesis (12, 15), we have measured the accumulation of oilbody proteins to define developmental relationships and to detect differential synthesis in response to regulatory factors.

MATERIALS AND METHODS

Plant Material

Microspores were obtained from rapeseed (*Brassica napus* cv Topas, Agriculture Canada, Saskatoon). Plants were grown in environmental growth chambers at 20/15°C (day/night) with 16 h day and a light intensity of 400 μmol m^{-2} s^{-1} using Grolux fluorescent lamps (GTE/Sylvania Drummondville,

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[2] Abbreviation: JA, jasmonic acid.

Quebec, Canada). Donor plants were maintained at this temperature until 4 to 5 weeks of age and then moved to a chamber with 15/10°C, 16 h day/8 h night.

Embryo Culture

Microspore isolation and culturing was performed as previously described using the 40% Percoll step gradient (15). At torpedo to cotyledonary stages of development, whole cultures were sieved through sterile nylon screens of either 500 or 250 μm mesh before labeling. A more homogeneous morphological population free of smaller embryos and dead cells was therefore retained by the screens. Globular and heart stage cultures were sieved on screens of 62 and 125 μm, respectively. They were replated with fresh half-strength NLN, *i.e.* modified Lichter medium (6). For the sorbitol osmoticum treatments, the NLN also contained 12.5% sorbitol (w/v).

Chemical Treatments

Stock 10 mM ABA (mixed isomers, Sigma) was made up in 50% DMSO, whereas JA and its methyl ester (mixed isomers, Apex Organics, Oxford UK) and GA_3 (Sigma) were solubilized in 95% ethanol as 50 mM stocks. Therefore, appropriate volumes of these stocks were added to embryo cultures for a final concentration of 10 or 30 μM. Controls received the appropriate volume of DMSO or ethanol.

Protein Synthesis and Analysis

These methods were essentially as reported (12). [^{35}S]Methionine (370 MBq/mL, 43.4 TBq/mmol, Amersham) was added to gravity-settled embryos in 15 mL sterile conical plastic tubes. Generally, a settled volume of 0.5 to 1.0 mL embryo mass in a total volume of 3 to 4 mL NLN medium was labeled with 0.75 to 1.5 MBq/mL [^{35}S]methionine. The tubes were placed lying flat in a closed container on a rotary shaker (60 rpm) at room temperature for 5 to 7 h or for shorter times as indicated in the figures.

After labeling, the embryos were transferred to a mortar on ice and rinsed with cold homogenization buffer (0.15 M Tricine-KOH, pH 7.5, 1.5 M NaCl, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 0.4 M sucrose). Before grinding in approximately 5 volumes of buffer, 2 mM PMSF, a pinch of acid-washed sea sand, and high molecular weight polyvinylpolypyrrolidone were added to the embryos. The resultant homogenate was filtered through one layer of Miracloth, the brei rehomogenized, and the filtrates pooled. The final brei in the filter was then hand squeezed to extract most of the liquid. In some experiments, total filtrate volume was estimated (usually 10 mL) and duplicate 10 μL aliquots were pipetted into tubes and precipitated with 2 mL of cold 10% TCA for calculating total homogenate protein [^{35}S]methionine incorporation. The homogenates, in 15 mL Corex tubes, were overlaid with 2 to 3 mL of the homogenization buffer containing 0.1 M sucrose. Samples were centrifuged at 7000*g* for 20 min in a Sorvall SS 34 fixed angle rotor at 4°C. The floating fat pad was transferred by loop and syringe to a new tube containing 0.4 M sucrose buffer, briefly vortexed, and overlaid with the 0.1 M sucrose buffer. The second centrifugation was at 18,000*g* for 20 min. This washing of the fat pad was repeated. The first 18,000*g* supernatant below the 0.1 M sucrose was also spun a second time at 18,000*g* and aliquots of this were precipitated with 4 volumes of cold acetone at −20°C overnight. The acetone precipitates were pelleted and N_2 air dried before solubilizing in 0.5% SDS for running on gels as the cytoplasmic-soluble proteins. The washed oilbody fraction was transferred to 1.5 mL Eppendorf tubes and centrifuged 2 to 3 times for 4 min at 10,000 rpm in a microfuge, withdrawing supernatant by syringe from under the lipid pad each time. Finally, the oil bodies suspended in 0.3 to 0.5 mL of buffer were extracted with an equal volume of acidic Bligh and Dyer reagent (choroform:methanol:KCl [2:1:0.8]) in which the KCl was 1 M in 0.2 M H_3PO_4. After vigorous vortexing, the samples were spun in the microfuge for 5 min at 10,000 rpm. This resulted in an upper aqueous phase, a lower chloroform phase, and a visibly precipitated interphase layer. All liquid was withdrawn, the tubes dried briefly by an N_2 stream, and the precipitate was solubilized in 100 μL of 0.5% SDS, boiled 5 min, and briefly centrifuged to pellet insoluble material. Aliquots (3–5 μL) of the oilbody or acetone-precipitated supernatant proteins were 10% TCA precipitated for at least 1 h at 4°C. They were then heated in a boiling water bath for 2 to 3 min, cooled, transferred onto glass fiber filters with suction, and washed with 15 mL of 5% TCA. Filters were then washed twice with 2 mL 95% ethanol, air dried, and counted in PCS II scintillation fluor (Amersham). Averaging duplicate dpm and correcting for original volume allowed calculation of total labeled TCA-precipitable counts in the oilbody fraction and total homogenate. Thus, estimated equivalent counts per well were loaded onto gels.

Gradient 8 to 15% SDS polyacrylamide slab gels were used for most of the results reported here except Figures 1 and 6, which were 15% polyacrylamide minigels. Protein samples were mixed with an equal volume of 0.1 M Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 8% glycerol, 0.3% bromphenol blue (Sigma), and boiled for 1 to 2 min. Gels were run at 20 mA until the dye front entered the separating gel, then run at 30 mA for 4 to 5 h until the dye front was off the gel. They were stained in 40% methanol, 10% acetic acid, 0.15% Coomassie Brilliant Blue R, destained, treated with En^{3}Hance (New England Nuclear), dried, and exposed to X-Omat AR (Eastman Kodak) film for 2 or more d at −80°C. Protein concentration determinations were made using the Pierce BCA reagent with BSA standards.

Antibody Production and Western Blotting

The 32 kD oilbody-associated protein was separated by preparative SDS-PAGE. After staining with Coomassie Brilliant Blue R and destaining, the gel was dried on 3MM paper. After drying the gels, the 32 kD polypeptide band was cut out and released from the 3MM paper by adding a few drops of water on the back side of the paper. The bands were frozen in liquid nitrogen and ground into a fine powder. The powder was suspended into 1.5 mL PBS (140 mM NaCl, 28 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.0) and mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected into female New Zealand white rabbits. At week 2, the rabbits were reinjected with the protein suspension and

an equal volume of Freund's incomplete adjuvant. After 6 weeks, total blood was collected and stored at 4°C overnight, then centrifuged at 5000*g* for 30 min. The serum was stored at −60°C in 500 μL aliquots. Protein samples were separated by 15% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (MSI, Fisher) by electroblotting for 3 h at 50 V in 25 mM Tris-Cl, 192 mM glycine, 0.05% SDS. The rest of the procedure was as previously described (12). For the primary antibody incubation, a 1:2000 dilution was used; for the secondary antibody (goat anti-rabbit-alkaline phosphatase linked) incubation, 1:3000 dilution was used.

Subcellular Fractionation

Proteins from either control torpedo stage embryos or those treated with 10 μM ABA for 24 h were fractionated and subjected to Western analysis using the antibody against the 32 kD oilbody-associated protein to determine subcellular distribution of the protein. Fractionation was performed essentially by the method of DuPont *et al.* (1) and included a sucrose-density gradient from which three interfaces were collected at 34 to 40%, 22 to 30% sucrose, and supernatant/22%. These three separated fractions are referred to notionally as plasmalemma, tonoplast, and ER as in ref. 1. A 1000*g* pellet obtained prior to the separation of a microsomal fraction was selectively lysed with detergent to yield either a lysed plastid or nuclear fraction. First, the 1000*g* pellet was treated with 0.5% (v/v) Triton X-100 and maintained on ice for 20 min. It was then recentrifuged at 3000*g* for 10 min. The supernatant was acetone-precipitated and called the "plastid" fraction. The 3000*g* pellet was then treated with 1% (v/v) sarkosyl and maintained at 4°C for 20 min. After centrifugation at 3000*g* for 10 min, the supernatant was acetone-precipitated and designated the "nuclear" fraction. It should be noted that the naming of these fractions is for convenience and reflects the enrichments reported by DuPont *et al.* (1) using the identical membrane separation techniques. Of these protein fractions, each acetone pellet was redissolved in small volumes of 0.5% SDS and the concentration determined using the Pierce BCA reagent with BSA as standard. Ten micrograms were loaded per well. The gel was electroblotted onto nitrocellulose membranes and treated with the antibody and secondary antibody linked to alkaline phosphatase as previously described (16).

RESULTS

Effect of Hormones and Osmoticum on Oilbody Protein Synthesis

Initially, we attempted to establish the specificity of response to phytohormones and to determine whether oilbody protein accumulation was sensitive to osmoticum, as is the case for many seed-specific proteins (7, 15). Figure 1 shows an autoradiogram obtained from an SDS polyacrylamide gel loaded with equal counts of a [^{35}S]methionine-labeled oilbody protein fraction obtained from torpedo stage microspore-derived embryos of *B. napus* (12). Even in the absence of any phytohormone treatment, the torpedo-stage embryos synthesize at least three oilbody proteins of approximate molecular masses of 19, 23, and 32 kD. With higher resolution, the 19 kD band is a 19 to 20 kD doublet and the 23 kD band is a doublet of proteins 22 to 23 kD in size. On treatment with 10^{-5} M ABA, there is a strong stimulation of production of the 19 and 23 kD proteins, but a decrease in the relative rate of synthesis of the 32 kD protein. Treatment with JA gave a concentration-dependent effect. At 10 μM JA, no change in oilbody protein profiles was detected, but at 30 μM there was an increase in synthesis of the 19 kD protein and a less pronounced effect on the 23 kD protein. Treatment with methyl jasmonate at 30 μM had no detectable effect on oilbody protein profiles. Application of GA_3 (10 μM) had little or no effect on 19 and 23 kD oilbody protein synthesis, although there appears to be a small stimulation of synthesis of the 32 kD protein. In addition to these treatments with plant growth substances, the embryos were treated with 12.5% sorbitol as an osmoticum. We have previously shown that this osmoticum strongly stimulates the expression of storage protein genes in microspore-derived embryos. Clearly, the synthesis of 19 and 23 kD oilbody proteins is also enhanced by the sorbitol treatment. It is interesting to note that both the sorbitol and ABA treatments reduce the relative proportions of the 32 kD protein.

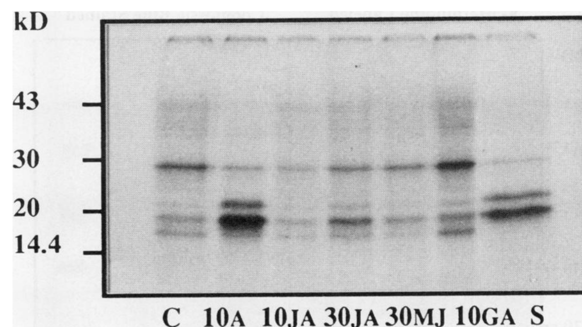

Figure 1. The effects of phytohormone and osmoticum treatments of microspore embryo cultures on the synthesis of oilbody proteins. Torpedo stage (19–20 d) microspore embryos in culture were treated for 3 d before labeling with 0.93 MBq/mL [^{35}S]methionine (42.6 TBq/mmol) for 6 h. Oilbodies were isolated and associated proteins were prepared for fluorography as described in "Materials and Methods." The lanes correspond to the following treatments: C, control containing 0.05% (v/v) DMSO; 10A, 10 μM ABA in 0.05% DMSO; 10JA, 10 μM JA; 30JA, 30 μM JA; 30MJ, 30 μM methyl jasmonate; 10GA, 10 μM GA_3; S, 12.5% (w/v) sorbitol. An estimated 10,000 dpm were loaded per well.

Time-Course of ABA-Stimulated Oilbody Protein Synthesis

Next, we obtained a time-course of the ABA effect on oilbody protein biosynthesis. For this, late torpedo/early cotyledonary embryos were collected and labeled with [^{35}S] methionine for a 2 h period after pretreatment with ABA for 0 to 8 h. Equal counts were loaded onto each well. The gel was stained with Coomassie brilliant blue to provide an estimate of the amount of protein in each labeled sample. The gel and fluorogram obtained in this experiment are shown in Figure 2. As can be seen in the left-hand panel of Figure 2, for loadings of equivalent incorporated counts there is in-

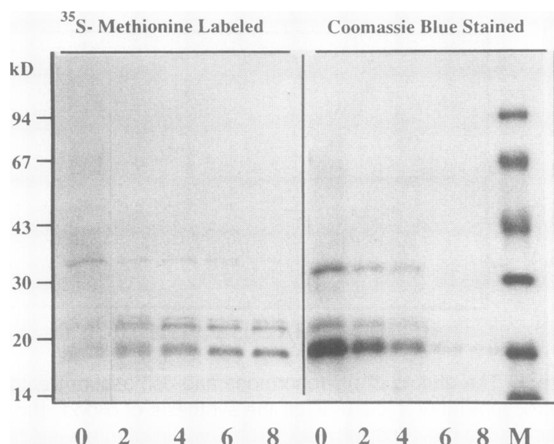

Figure 2. Time course of the ABA effect on oilbody protein synthesis. *B. napus* cv Topas early cotyledonary embryos (23 d) collected after sieving on 500 μm nylon screens were labeled with 1.11 MBq/mL (lanes 1–3) or 1.85 MBq/mL (lanes 4 and 5) [^{35}S]methionine (42.6 TBq/mmol) for 2 h. Lane 1 is a control that was labeled between 2.5 and 4.5 h after sieving and transfer to test tubes. ABA (10 μM) was added to samples corresponding to lanes 2 through 5 at zero time and then [^{35}S]methionine labeled at 2 h intervals as follows: lane 2, 0 to 2 h; lane 3, 2 to 4 h; lane 4, 4 to 6 h; lane 5, 6 to 8 h. The left panel shows the fluorogram of the time course in which an estimated 10,000 dpm were loaded per well. The right panel shows the Coomassie brilliant blue-stained gel photographed after drying down for fluorography.

creased synthesis of the 19 and 23 kD oilbody proteins after only 2 h. The adjacent picture of the Coomassie brilliant blue-stained gel (Fig. 2, right-hand panel) demonstrates the drastic change in specific activity of the label in the presence of ABA. From these same samples, more precise measurements of the specific activity of the labeled proteins were obtained by protein estimation and scintillation counting. The results of these estimates are given in Table I. This shows that over the 8 h period there is more than a 25-fold increase in specific activity of labeling, which reflects a substantial increase in rates of synthesis.

Time-Course of Osmoticum Stimulation of Oilbody Protein Synthesis

Given the rapid response in oilbody protein synthesis of these embryos to ABA treatment, it was of interest to determine how rapidly these proteins responded to osmotic stress. We have previously shown that within the first 4 h of osmotic stress, these embryos attain a sixfold increase in ABA levels (15). If ABA acts as an intermediary in the osmotic response, we might predict a response to osmoticum that lags behind the change in endogenous ABA levels. Therefore, we measured the change in synthesis of oilbody proteins after treatment with 12.5% sorbitol. Figure 3 shows the results of [^{35}S] methionine incorporation 0, 6, 9, and 12 h after the start of the osmotic stress. We also performed a 12 h nonstressed control to detect any changes associated with replenishing the medium rather than those due to the osmotic stress. As can be seen in Figure 3, there is only a small response to osmoticum during the first 6 h, but this increases substantially from 6 to 9 h. Lane 5 of Figure 3 suggests that there is a small effect simply due to replenishing the medium (independent of osmoticum). When this is taken into account, significant increases in oilbody protein synthesis are not detected until the 6 to 9 h sample.

Table I. *Increase in Specific Activities of Oilbody Proteins Labeled at Times after Addition of ABA to Microspore Embryos*

Data are from 23-d early cotyledonary embryos shown in the fluorogram of Figure 2 (left-hand panel). Samples indicate the timing of the 2 h period of [^{35}S]methionine labeling after addition of 10 μM ABA to the cultures.

Sample	Protein	dpm/μL	dpm/μg Protein
	μg/mL		
0–2 h	1.36	273	201
2–4 h	0.60	343	572
4–6 h	0.63	2131	3382
6–8 h	0.51	2984	5851

Developmental Sensitivity of Oilbody Protein Synthesis to ABA

Although it is clear from the above that synthesis of these proteins is both ABA- and osmoticum-sensitive, it was not clear at what developmental stages these sensitivities occur. Therefore, we subjected microspore-embryo cultures at globular, heart, torpedo, and cotyledonary stages to ABA treatments (10^{-5} M) to determine [^{35}S]methionine incorporation into oilbody proteins. Surprisingly, and unlike the storage

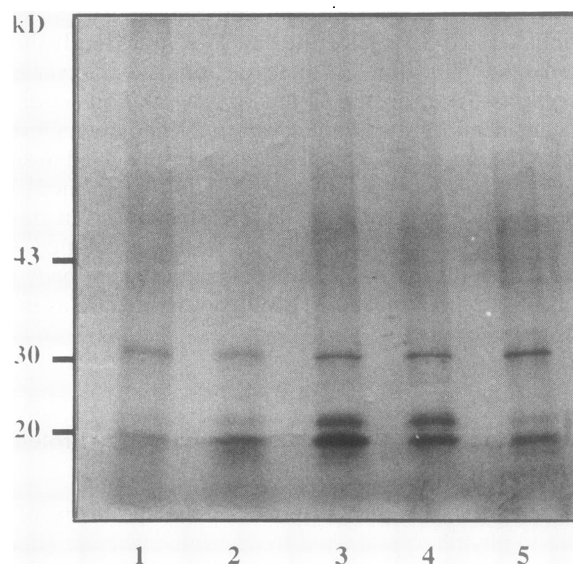

Figure 3. Time course of oilbody protein synthesis after sorbitol treatment. Cotyledonary embryos (22 d) collected on 500 μm screens were labeled with 1.22 MBq/mL [^{35}S]methionine for 3 h time intervals. Lanes 1 and 5 are controls labeled at 3 to 6 and 9 to 12 h after transfer to fresh medium, respectively. Medium containing 12.5% sorbitol was added to the remaining embryos for methionine labeling at 3 to 6 (lane 2), 6 to 9 (lane 3), and 9 to 12 h (lane 4) after transfer. 10,000 dpm per lane were loaded onto the gel for fluorography.

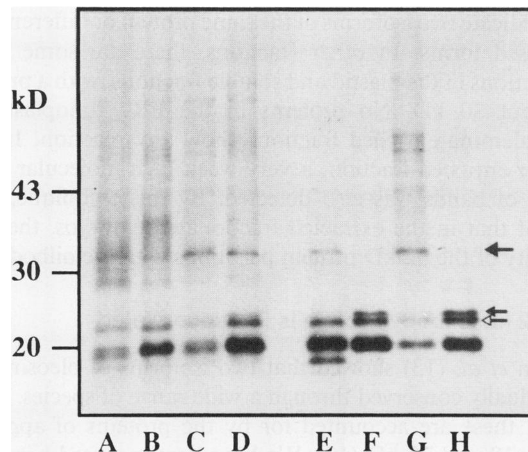

Figure 4. Developmental sensitivity of oilbody protein synthesis to applied ABA. An estimated 10,000 dpm were loaded per well for paired samples of controls (lanes A, C, E, G) and ABA-treated (lanes B, D, F, H). All samples were treated for 2 d with ABA, then labeled for 4 h with 1.85 MBq/mL [^{35}S]methionine. Lanes A and B, 10-d-old cultures, sieved on 62 μm screens to obtain globular embryos. Lanes C and D, 13-d-old cultures, sieved on 125 μm screens to obtain heart stage embryos. Lanes E and F, 17-d-old cultures, sieved on 250 μm screens to obtain torpedo to early cotyledonary embryos. Lanes G and H, 25-d-old cultures, sieved on 500 μm screens to obtain cotyledonary stage embryos.

proteins of *B. napus* (15), sensitivity to ABA was found at very early stages of embryogenesis. Figure 4 illustrates this point by the comparison of alternate lanes (with or without ABA) and the four developmental stages: globular (lanes A, B), heart (lanes C, D), torpedo (lanes E, F), and cotyledonary (lanes G, H). In all cases, the embryos respond to 10^{-5} M ABA with an increase in 19 and 23 kD oilbody proteins. The decrease of the 32 kD protein only occurs at the torpedo and cotyledonary stages. Table II shows the relative change in [^{35}S]methionine incorporation into total oilbody proteins at each developmental stage on treatment with ABA. This indicates that there is an approximately eightfold stimulation of oilbody protein synthesis after ABA treatment even in globular stage embryos.

Table II. *Changes in Relative [^{35}S]Methionine Incorporation into Oilbody Protein as a Percentage of Total Protein in the Presence or Absence of ABA According to Embryo Developmental Stage*

Values are total oilbody protein ^{35}S-dpm as percentage of total respective homogenate ^{35}S-dpm estimated from TCA-preciptable material. The fluorogram of these samples is shown in Figure 4.

Treatment	Relative [^{35}S]Methionine Incorporation			
	10–12 d globular	13–15 d heart	17–19 d torpedo	25–27 d cotyledonary
− ABA	0.044	0.064	0.120	0.89
+ ABA	0.337	0.170	0.960	2.63
-Fold increase	7.7	2.6	8.0	2.9

Effects of Heat Shock on Oilbody Protein Synthesis

In order to determine whether the production of oilbody proteins might also be affected by other environmental stresses, we subjected the embryos to heat shock using a 43°C treatment for 3 h. Figure 5 demonstrates that the 43°C treatment does indeed elicit typical heat shock response, shutting down much of the synthesis of cytoplasmic proteins while greatly increasing the synthesis of certain heat shock proteins. The presence of ABA has no effect on this response. Examination of the [^{35}S]methionine-labeled oilbody fraction reveals perhaps a small effect on the synthesis of the 19 kD proteins, but it is marginal compared with the effect of 10^{-5} M ABA. It is noteworthy that whereas synthesis of many cytoplasmic proteins is arrested upon heat-shock, the oilbody proteins continue to be synthesized and retain their sensitivity to ABA, although absolute amounts synthesized after heat shock are reduced about 10-fold (Fig. 5).

Cellular Localization of the 32 kD Oil Body-Associated Protein

In addition to the major protein bands (19 and 23 kD) associated with oil bodies in *Brassica* embryo extracts, normally even in high-salt washed oilbodies higher molecular mass bands may be found. Most of these bands cross-react

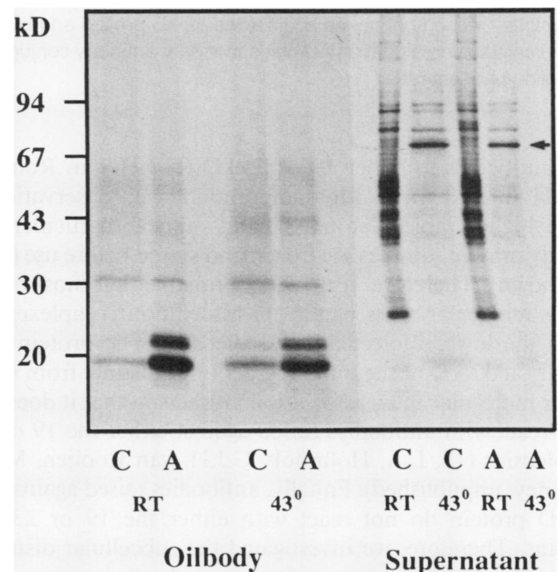

Figure 5. The effect of heat shock on cytoplasmic and oilbody protein fractions of microspore embryos with or without ABA treatment. Cotyledonary stage embryos (25 d) without (lanes C) or with (lanes A) a 2 d ABA treatment (10^{-5} M) were placed at 43°C for 3 h, then washed and replated in fresh NLN medium. Both controls (RT) and heat-shocked (43°C) samples were incubated for a further 14 h at 25°C with 2.8 MBq/mL [^{35}S]methionine. After being centrifuged twice for 20 min at 18,000*g*, homogenate supernatant proteins were acetone precipitated and later SDS solubilized. These are seen in the right-hand panel; oilbody proteins are shown on the left-hand panel. In both panels, C, ABA-free; A, ABA-treated; RT, room temperature. 43° indicates all samples treated for 3 h. The arrow indicates an approximate 78 kD heat shock protein.

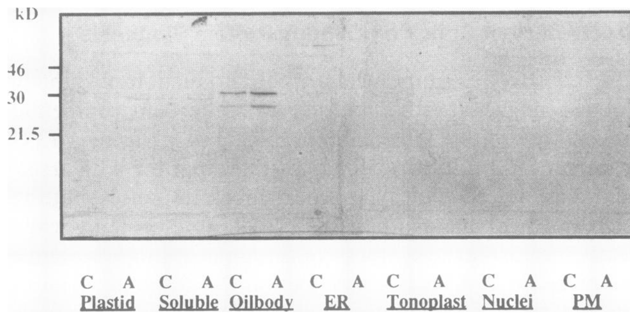

Figure 6. Subcellular localization of the 32-kD, oilbody-associated protein after centrifugal fractionation of total cellular homogenates of torpedo stage, microspore-derived embryos of *B. napus* cv Topas and application of the microsomal pellet onto a sucrose step-gradient (1). The gradient contained interfaces at 34 through 40% (labeled PM, plasma membrane-enriched), 22 through 30% (labeled Tonoplast) and supernatant through 22% (labeled ER, endoplasmic reticulum-enriched). Other organellar fractions (plastid- and nuclei-enriched) were obtained by differential lysis of a 1000*g* pellet using Triton X-100 or Sarkosyl (see "Materials and Methods" and ref. 1). (Membrane fraction designations are notional, based not on marker enzymes, but on corresponding fractions described by DuPont *et al.* [1]). Soluble proteins were derived from the 100,000*g* supernatant. Oilbodies were prepared as in "Materials and Methods." Lanes marked C and A correspond to untreated and ABA- (10 μM) treated embryos from the same original preparation. An estimated 10 μg was loaded per lane and was run into 15% polyacrylamide gels that were then electroblotted onto nylon membranes. The blot was treated with a polyclonal antibody obtained against the 32 kD protein and binding was revealed using a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase.

with antibodies to either 19 or 23 kD (G.J.H. van Rooijen, L.A. Holbrook, M.M. Moloney, unpublished observations). These higher molecular mass bands appear particularly if oilbody protein samples are frozen and stored before use (data not shown). Therefore, it is highly probable that most of the higher molecular mass bands are association complexes between the lower molecular mass oleosins. The protein that appears at 32 kD in the oilbody fraction is distinct from these higher molecular mass association artifacts in that it does not cross-react with antibodies raised against either the 19 or 23 kD oleosins (13, L.A. Holbrook, G.J.H. van Rooijen, M.M. Moloney, unpublished). Equally, antibodies raised against the 32 kD protein do not react with either the 19 or 23 kD oleosins. Therefore, we investigated the subcellular distribution of the 32 kD protein in fractionated cellular extracts of *B. napus* microspore embryos to determine whether it was uniquely found in oilbody preparations. This was done by Western blotting of polyacrylamide gels loaded with equivalent amounts of protein from bands on a 80,000*g* step-gradient (1). The results of this fractionation are shown in Figure 6. The fractionation was performed on untreated and ABA-treated (10 μM) embryos, and fractions representing enrichments for plastid, ER, tonoplast, plasma membrane, and nucleus, as well as the soluble fraction, were prepared. Clearly, the only strong reaction that occurs is with the oilbodies. With this antibody, two bands of about 32 and 27 kD are detected, the stronger being the 32 kD band. These may indicate two isoforms of the same protein or differentially processed forms. In other fractions, there are some weak interactions in the plastid and soluble fractions, with a protein of about 30 kD. No proteins in the ER-, tonoplast-, or plasmalemma-enriched fractions show any reaction. In the nuclear-enriched fraction, a very weak high-molecular mass ladder of bands was also detected. By this technique, it is evident that in the extracted fractionated embryos, the vast majority of the 32 kD protein partitions with the oilbodies.

The 32 kD Oilbody Protein is Phosphorylated

Tzen *et al.* (13) showed that two isoforms of oleosins are individually conserved through a wide range of species. In *B. napus,* these are accounted for by the proteins of approximately 19 and 23 kD (13). We have demonstrated here that the 32 kD protein of rapeseed oilbodies is a different class of protein, at least on the basis of its regulation (see Figs. 1, 2, and 4). Unlike the 19 and 23 kD proteins, after ABA treatment the 32 kD protein is less abundant than in the controls except during early stages of embryogenesis. Therefore, we performed experiments to determine any additional differences between this protein and the oleosins of *B. napus.* One clear difference is that the 32 kD oilbody protein is capable of undergoing phosphorylation *in vivo,* as shown in Figure 7. In this experiment, torpedo/early cotyledonary microspore embryos were labeled with either [^{35}S]methionine (lanes 1

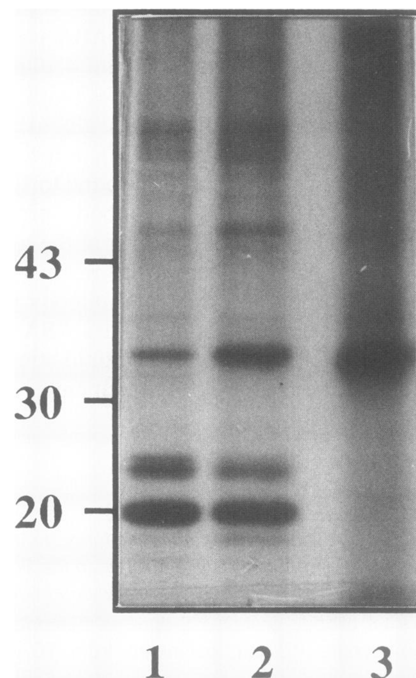

Figure 7. Phosphorylation of the 32 kD oilbody protein. Torpedo to early cotyledonary embryos (24 d in culture) were labeled with [^{35}S] methionine (lanes 1 and 2) or with [^{32}P]orthophosphate (lane 3). Oilbody proteins were isolated from these embryos and were run on the same gel. [^{35}S]Methionine-labeled control (lane 2) and [^{35}S]methionine-labeled 2 d ABA treatment (lane 1); [^{32}P]orthophosphate-labeled oilbody proteins from ABA-untreated embryos (lane 3).

and 2) or [^{32}P]orthophosphate. This preparation shows the typical profile of oilbody proteins produced in the presence or absence of ABA, but when a phosphate label is introduced, it is clear that the only oilbody protein subject to phosphorylation is the 32 kD protein (Fig. 7, lane 3). The phosphorylation reaction itself appears to be unaffected by ABA (data not shown), although overall synthesis of the 32 kD protein is reduced during the period of labeling. This protein is clearly different from the oleosins both in regulation and posttranslational processing.

DISCUSSION

Using microspore-derived embryos, we have characterized some of the factors regulating the synthesis of oilbody proteins in *B. napus*. The *Brassica* oilbody proteins comprise a series of proteins between 19 and 24 kD that appear to be oleosin isoforms (13). In addition, a 32 kD protein appears to be of a different class on the basis of immunological properties (13), regulation, and posttranslational modification. The factors that regulate the synthesis of these proteins include ABA, jasmonates, and osmotic stress. Specificity of the regulation is supported by the lack of effect of either GA_3 or heat shock. Accumulation of 19 and 23 kD proteins is enhanced by ABA, JA, and osmotic stress. Synthesis of the 32 kD protein is somewhat reduced in the presence of ABA, but to a lesser extent with JA (16). We have recently shown that JA is a natural product of *Brassica* and *Linum* embryos and, thus, it could be one of the factors involved in oleosin regulation *in vivo* (16). Sorbitol treatment, which has a large effect on 19 and 23 kD oleosin synthesis, also down-regulates the 32 kD protein.

Time courses of the ABA and osmotic effects are consistent with the hypothesis that osmotic stress provokes increased levels of ABA (15) and possibly other regulators (16) that in turn stimulate the synthesis of the oleosins. This result is similar to our findings for the regulation of storage protein gene expression in this system (15). The stimulation of synthesis of the oleosins by ABA, JA, or osmoticum is at least in part explained at the transcriptional level as all three of these stimuli provoke increases in oleosin mRNA accumulation (G.J.H. van Rooijen, R.W. Wilen, L.A. Holbrook, M.M. Moloney, submitted).

Although it is tempting to consider regulation of oleosin gene expression as being very similar to that of storage proteins, there are some major differences. This is exemplified by the developmental sensitivity of oleosin gene expression and protein accumulation to ABA. We recently reported that in globular microspore-derived embryos of *B. napus*, expression of napin was undetectable until heart stage and then only in the presence of ABA (15). In this paper, we show that globular embryos are capable of accumulating oleosins and the 32 kD oilbody protein. Furthermore, even at this stage, ABA sensitivity has already developed (Fig. 4). This early sensitivity to ABA is also reflected at the transcriptional level (G.J.H van Rooijen, R.W. Wilen, L.A. Holbrook, M.M. Moloney, submitted). This result is intriguing given the report by Murphy *et al.* (8) on *B. napus* zygotic embryos. These workers showed that the 19 kD oleosin only accumulated quite late in embryogenesis and lagged several weeks behind both napin and cruciferin during seed development. The differences might be in part explained by the different systems: zygotic *versus* microspore embryos; field grown *versus* growth chamber-grown material. However, it is noteworthy that a comparison of zygotic and microspore-derived embryos showed that oleosin transcripts were easily detectable in late heart stage zygotic embryos (G.J.H. van Rooijen, R.W. Wilen, L.A. Holbrook, M.M. Moloney, submitted) from plants raised in growth chambers.

Among the complement of oilbody-associated proteins, the 32 kD protein is distinct. We have demonstrated here that it is unique among the oilbody proteins in that it is phosphorylated. There is insufficient information yet to assign a role to this protein, which is present in unstressed or non-ABA-treated embryos and which declines in abundance during zygotic embryogenesis (data not shown). Initially, we thought that this protein might be a *Brassica* homolog to a 34 kD oilbody protein found in soybeans (5). The 34 kD soybean protein is distinguishable from the soybean oleosins immunologically, by amino acid sequence and by hydrophobicity profiles. It undergoes processing on germination by cleavage of its N-terminal end to yield a 32 kD protein (3). However the soybean 34 kD protein appears to accumulate only in cotyledonary-stage embryos. This is different from the regulation found here for the *Brassica* 32 kD oilbody protein, which is detected at very early stages of embryo development. This, combined with our demonstration that the *Brassica* 32 kD protein is phosphorylated, suggests it is not equivalent to the soybean 34 kD protein. In spite of this distinction, an important caveat should be mentioned. Recent work (E.M. Herman, personal communication) has shown that the oilbody localization of the 34 kD soybean protein is artifactual. Immunocytolocalization suggests that this protein is probably localized in the protein body *in vivo*. The reasons for such specific partitioning to oilbodies upon extraction are not clear, but this does raise the possibility that other proteins in different systems could associate with oilbodies during extraction. We are investigating this possibility by immunolocalization of the *Brassica* 32 kD protein in developing microspore-derived embryos. Given the apparent down-regulation of the 32 kD protein toward seed maturity and the capacity of this protein for phosphorylation, we are also studying the possibility that this protein is implicated in oilbody biogenesis or oleosin targeting.

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