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SYNTHESIS OF STORAGE RESERVES IN DEVELOPING SEEDS OF SUNFLOWER

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Abstract—The patterns of synthesis and accumulation of storage compounds were compared in young developing embryos of sunflower. Oil biosynthesis was clearly initiated very early in development with total lipids accounting for about 18% of the dry weight at the earliest stage studied. This was associated with the accumulation of oleosins and the expression of cytochrome b_5. In contrast, significant amounts of helianthinin (11S globulin) and 2S albumin were only observed in stages 3 and 4, respectively. The early expression of oleosins contrasts with previous studies of other oilseeds and is consistent with the hypothesis that they are intimately associated with the biogenesis of oil bodies.

INTRODUCTION

Sunflower seeds accumulate two major groups of storage compounds, triacylglycerols (oil) and proteins. The storage proteins in turn comprise two groups, the 11S helianthinin which belongs to the widely distributed legumin-like family of globulins [1] and the 2S albumins which again belong to a larger protein family [2]. The helianthinins are generally considered to be the major group of storage proteins, being reported to account for some 60% of the total proteins in the mature seed with the 2S albumins accounting for about 20% [3]. However, early studies of the salt-soluble proteins by sucrose gradient centrifugation gave different results, with 62% 2S albumins and 38% 11S globulins [4].

In addition to the storage albumins and globulins, sunflower seeds also accumulate a third group of proteins. These are the oleosins, or oil body proteins, which are associated with the outer surface of the oil bodies and are thought to act as an emulsifying agent to prevent coalescence. Murphy and co-workers have reported that oleosin synthesis occurs some time after the initiation of oil and storage protein synthesis in embryos of rape and coriander [5–8], but this has been disputed by Napier *et al.* [9] who suggested that an earlier initiation of oleosin synthesis is consistent with a role in oil body biogenesis. A previous study of sunflower showed the presence of oleosin mRNAs in late stage embryos [10] but subsequent work from our laboratory demonstrated the presence of two distinct groups of oleosin transcript which showed early and late patterns of expression, respectively, in the developing sunflower seed [11]. In the present report we demonstrate that oleosins are present in very young developing embryos of sunflower, before the initiation of storage protein synthesis, which is consistent with a role in the biogenesis of oil bodies.

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RESULTS AND DISCUSSION

In order to compare the patterns of storage product accumulation during the early stages of embryo development, seeds were harvested from the outermost whorls of heads and the embryos separated into groups on the basis of fresh weight. Seven developmental stages were selected for analysis (see legend to Fig. 1), the most advanced of which (stage 7, 65–70 mg fr. wt) had dry wts of about 18–20 mg compared with dry wts of 65–70 mg for mature embryos.

SDS-PAGE of total proteins from mature embryos (Fig. 2A) showed three groups of polypeptides corresponding to the large (α and α^1) subunits of helianthinin (M_r 30–40,000), the small (β) subunits of helianthinin (M_r about 20–25,000) and the 2S albumins (M_r about 10–15,000). The identities of these groups were confirmed by analysis of pure albumin and globulin fractions prepared from mature seeds

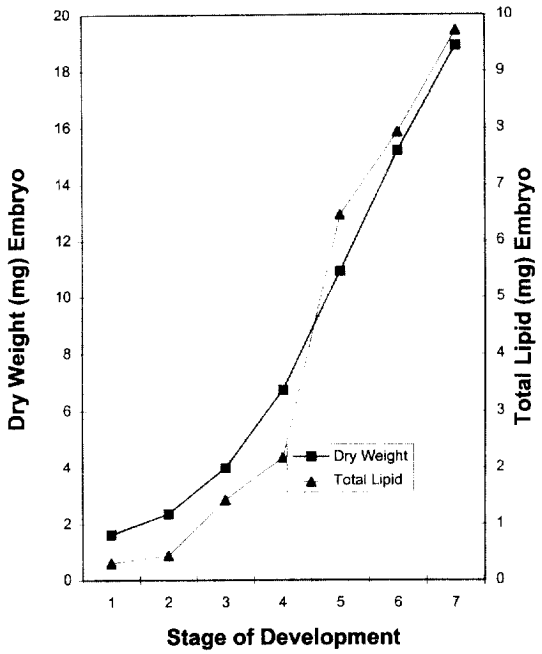

Fig. 1. The mature embryos varied in fresh weight from 95–100 mg and had a mean dry weight of 68.2 mg and a mean total lipid content of 38.6 mg. Mean dry weights and total lipid contents of sunflower embryos at 7 developmental stages. The developmental stages are defined on embryo fresh wt: 1, 10–15 mg; 2, 15–20 mg; 3, 25–30 mg; 4, 35–40 mg; 5, 45–50 mg; 6, 55–60 mg; 7, 65–70 mg.

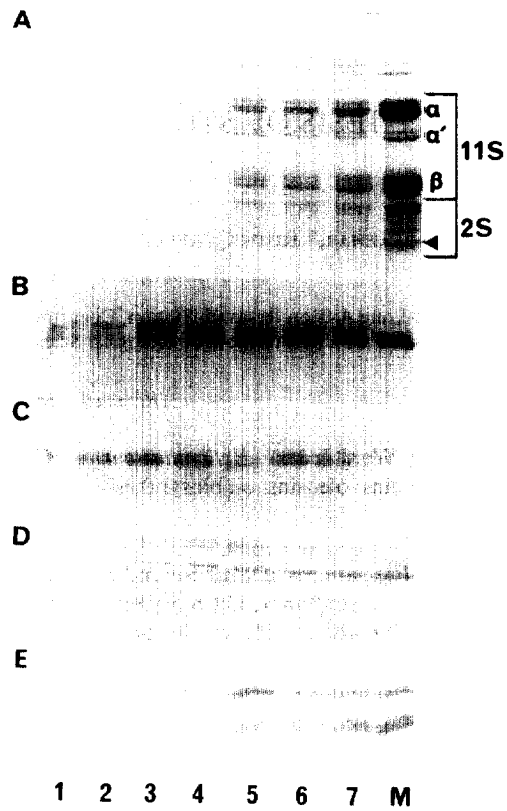

Fig. 2. A, SDS-PAGE of total proteins extracted from developing (stages 1–7) and mature (M) sunflower embryos. The patterns of 11S globulin (helianthinin) and 2S albumin subunits are similar to those reported by other workers [17] and the helianthinin subunits are accordingly labelled α, α^1 and β. The arrowhead indicates the methionine-rich 2S albumin SFA8. B–E, Western blots of the samples separated in part A, using antisera to B, sunflower oleosin; C, cauliflower cytochrome b_5; D, the large subunit of helianthinin; E, total 2S albumins of sunflower. Parts A–D were separated under reducing conditions and part E without reduction.

(see Experimental). The albumin and globulin fractions accounted for about 5.7% and 11.2%, respectively, of the seed dry wt. Similarly, co-electrophoresis with purified protein allowed the identification of the methionine-rich 2S albumin component called SFA8 [3] (see arrowhead in Fig. 2A).

The patterns of accumulation of 11S globulin and 2S albumin during seed development were determined by western blotting using specific polyclonal antisera (Fig. 2, D and E) while the populations of corresponding mRNAs were determined by northern blotting (Fig. 3, D, E). Messenger RNAs for helianthinin and 2S albumin were first detected in stage 3 and 4 embryos, respectively, with traces of protein being detected slightly earlier. This small difference may result from the western blotting procedure being less specific than the northern blotting. This pattern of storage protein synthesis agrees with the previous report of This *et al.* [12] who showed that the synthesis of helianthinin and of albumin subunits of M_r 12,500 and 15,000 was initiated at about 14–16 and 18–20 days after flowering, respectively. However, these authors also described an M_r 18,000 "albumin" which showed an earlier pattern of expression (11 days); this protein was not observed in the present study.

In contrast to the synthesis of storage proteins, the synthesis of lipids and oleosins was initiated very early in development. The stage 1 embryos contained almost 20% total lipids and this was accompanied by the presence of cytochrome b_5 (Fig. 2C), a small heme protein which plays a role in the desaturation of fatty acids for triacylglycerol synthesis [13]. It should be noted that cytochrome b_5 was not detected in the mature seeds, which is consistent with its enzymic rather than storage function.

Oleosin protein was also present throughout development (Fig. 2B) suggesting an intimate association with storage oil biosynthesis. This was confirmed by northern blotting which also confirmed our previous report that two sub-families of oleosin transcript are present which show early (detected by the probe pSOS, Fig. 3B) and late (detected by the probe pSOM, Fig. 3C) patterns of expression [11].

The demonstration of oleosin biosynthesis in early stages of development contrasts with several previous studies of oilseed rape [5, 6, 10] and *Coriander* [8]. These showed that oleosin synthesis was not concomitant with oil biosynthesis and only occurred after the initiation of storage protein synthesis. The reason

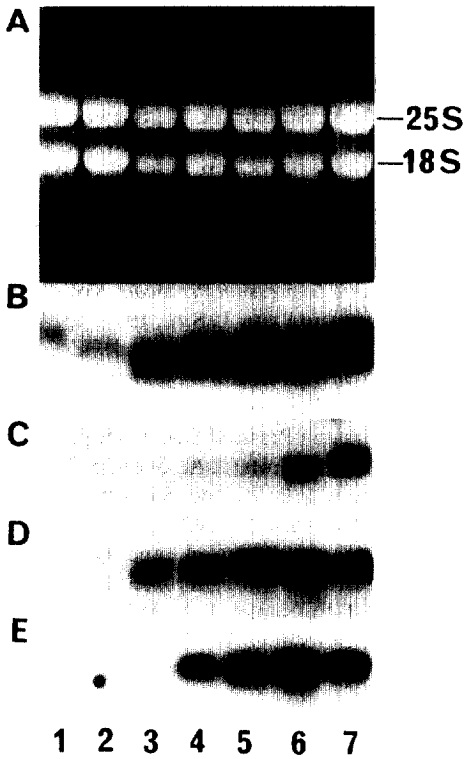

Fig. 3. Northern blotting of RNA fractions extracted from developing (stages 1–7) sunflower embryos. A, Gel before transfer stained with ethidium bromide to show the 18S and 25S ribosomal RNAs. B–E, Northern blots with probes for B, early oleosin (pSO5) [11]; C, late oleosin (pSOM) [11]; D, helianthinin (pHA6) (unpublished results of P. Thoyts, J. A. Napier and P. R. Shewry) and E, 2S albumin (pHAO) [18].

for this discrepancy is not known but we believe that the early expression of oleosins in sunflower is consistent with their role in stabilizing nascent oil bodies [9].

EXPERIMENTAL

Materials

Sunflowers (*Helianthus annuus* L. Dwarf Sunbred 246) were grown in the greenhouse and heads were tagged at anthesis. Seeds were collected from the outer whorl of the inflorescence and dissected to remove the embryos which were weighed, frozen in liquid N_2 and stored at −80 . Duplicate samples of 10 embryos of each stage were freeze-dried for dry wt determination. The means of these two determinations are presented in Fig. 1.

Protein and lipid analysis

Mature seeds were dehusked, ground and defatted with petrol (40–60). Albumins and globulins were extracted with 0.02 M Tris HCl buffer, pH 7.8, containing 0.5 M NaCl and 1 mM phenylmethylsulphonylfluoride. Globulins were precipitated from the supernatant by the addition of 1.5 vol. of ice cold MeOH followed by 3 vol. of ice cold acetone to precipitate albumins. Protein precipitates were freeze-dried and weighed.

Total protein was extracted from 20 mg defatted ground seed with 100 μl of SDS sample buffer (100 mM Tris HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS and 0.01% (w/v) bromophenol blue). SDS-PAGE using a modified Laemmli system and immunoblotting were carried out as described previously using goat anti-rabbit alkaline phosphatase conjugate (Sigma) as a detection system [14, 15]. Polyclonal antisera to cauliflower cytochrome b_5 [16], sunflower oleosin [11], sunflower 2S albumins (unpublished results of I. Anisimova, R. Fido and P. R. Shewry), and the large subunit of helianthinin (unpublished results of M. Millichip, A. K. Stobart, A. S. Tatham and P. R. Shewry) were used at dilutions of 1:100, 1:400, 1:1000 and 1:25,000 respectively.

Lipids were extracted from 50 mg samples of freeze-dried ground embryos by grinding sequentially with 1 ml aliquots of 2-PrOH, 2:1 (v/v) 2-PrOH:$CHCl_3$ and $CHCl_3$. The supernatants were combined, mixed with an equal vol. of 0.7% NaCl and the lower ($CHCl_3$) phase removed. The upper (aq.) phase was washed with 1 ml $CHCl_3$ and the two $CHCl_3$ phases bulked, reduced to dryness and weighed to determine total lipid.

RNA analysis

Total RNA was prepared from 0.5 g aliquots of embryos using Qiagen columns and following the manufacturer's protocol. RNA was separated on a denaturing agarose gel (1.2% (w/v) agarose, 6.5% (v/v) HCHO, 1 × MOPS buffer). 10 μg RNA (8 μl) was mixed with loading buffer (50% (v/v) $HCONH_2$, 6.5% (v/v) HCHO, 1 × MOPS, 1 μl of 1 mg ml ethidium bromide) and denatured at 65 for 15 min and cooled on ice. Electrophoresis was performed at 150 V in 1 × MOPS. The RNA was then transferred to Hybond-N nylon membranes by capillary action overnight, using Na-Pi buffer, pH 7.5 (10mM Na_2HPO_4, 1 mM EDTA). Filters were cross-linked by UV transillumination.

The membrane was pre-hybridised for 30 min at 65 in hybridisation buffer (7% SDS, 0.5 M Na_2HPO_4, 1 mM EDTA). The DNA was labelled using the Stratagene "Prime It" Kit, following the manufacturer's instructions. The probe was mixed with fresh hybridisation buffer and hybridisation was carried out at 65 overnight. The membranes were then washed with 2 × SSC, 0.1% (w/v) SDS for 30 min and then with 0.2 × SSC, 0.1% SDS for 30 min. The filters were exposed to the film for a day at −80 .

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