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Precociously germinating rapeseed embryos retain characteristics of embryogeny

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Abstract. We compared the germination of *Brassica napus* L. embryos at three stages of development – mid-cotyledon, maturation and mature dry – to determine at which stage they acquired the capacity for normal germination and seedling development. Embryos were removed from the seed and cultured on hormone-free medium, allowing them to germinate. The transition from embryogeny to germination was monitored both morphologically and biochemically, using synthesis of 12 S storage protein as a marker of embryogeny. The mature embryos (dry seeds) set the standard for normal seedling development: radicle emergence, hypocotyl extension and cotyledon expansion occurred within 2 d and true leaves were formed within a week of germination. Rocket immunoelectrophoresis indicated that the storage proteins in seedlings from mature dry embryos were completely degraded within a week. In contrast, the mid-cotyledon-stage embryos appeared to germinate abnormally, retaining many embryonic characteristics. Although the roots emerged, the hypocotyls did not elongate and secondary cotyledons instead of leaves were formed at the shoot apex. Also, the seedlings continued to synthesize and accumulate storage proteins. The maturation-stage embryos did develop into normal-looking seedlings, but complete degradation of storage proteins required several weeks, presumably reflecting continued synthesis and turnover. We conclude that embryogenic and germination-specific processes can occur concurrently and that the capacity to develop as normal seedlings is acquired gradually during the maturation process.

Key words: *Brassica* (precocious germination) – Embryo maturation – Germination (seeds) – Storage protein – mRNA (storage protein).

Abbreviations: dpa = days post anthesis; EDTA = ethylenediaminetetraacetic acid; FW = fresh weight

Introduction

Maturation is the final phase of seed development in most angiosperms. During this phase, embryogenic development ceases as the seeds dry down and enter a period of developmental arrest. In species which do not enter true dormancy, subsequent imbibition is sufficient to allow germination, and following this, the seedlings exhibit a pattern of growth dramatically different from that seen in embryogeny. While maturing embryos grow by overall cell expansion and accumulate high levels of storage reserves, growth in germinating seedlings is localized in the meristem and the seedlings use the storage products as a nutrient source until they develop the ability to support their growth by photosynthesis. Although embryogeny and germination are normally separated temporally by a period of developmental arrest, immature embryos of many species, when excised from the seeds and placed on basal culture medium, germinate precociously. *Phaseolus vulgaris* embryos placed in culture as early as the heart stage can germinate and develop into normal seedlings. However, younger embryos germinate at reduced frequencies, require longer periods to complete germination and their cotyledons remain small relative to mature embryos (Walbot et al. 1972). Long et al. (1981) found that immature *P. vulgaris* axes completed embryonic maturation in culture before switching to a typical germination pathway. Using carboxypeptidase C as a germination-specific marker, Ihle and Dure (1969) showed that immature cotton embryos began to germinate within a day of being placed in culture. The results of these studies indicate that the desiccation phase of embryo development may not be essential for maturation.

To determine the stage at which *B. napus* embryos acquire the capacity for normal germination and seedling development, i.e. become mature, ex-

cised embryos were placed in culture at different stages of development and germination was monitored both morphologically and biochemically. Storage-protein synthesis was used as a biochemical marker of continued embryo-like growth. *Brassica napus* embryos synthesize two major storage proteins: a "legumin-like" 12 S protein, which we will call cruciferin to distinguish it from the 12 S seed proteins of other species, and a 1.7 S basic protein, napin (Crouch et al. 1983). Cruciferin is composed of several polypeptides (Bhatty et al. 1968; Goding et al. 1970; Crouch and Sussex 1981) and constitutes 50–60% of embryo protein at seed maturity (Finlayson 1976). During embryo development within seeds, cruciferin is first detected at the early cotyledon stage and its accumulation continues until seed maturity (Crouch and Sussex 1981). Thus, cruciferin accumulates during the cell enlargement phase of embryogenesis, after cell division has ceased (Ching et al. 1974). Previous studies have shown that storage-protein synthesis in precociously germinating early-cotyledon-stage embryos decreases to very low levels within a few days, but does not cease completely (Crouch and Sussex 1981). The present work traces the development of precociously germinating embryos through much longer culture periods.

Material and methods

Plants. Seeds of *B. napus* L. cv. Tower (from Dr. W.D. Beversdorf, University of Guelph, Ont., Canada) were planted in plastic flats in a 2:1:1 (by vol.) mixture of soil, vermiculite and perlite (Krum, Silbrico Corp., Hodgkins, Ill., USA) and grown in window boxes for two weeks. The seedlings were then transplanted to 15 cm (6-inch) pots and grown under greenhouse conditions, in natural light supplemented with light from metal arc lamps (400 W Lucalox high-pressure sodium lamps; General Electric, Cleveland, O., USA) to give 16 h days, 3250–4850 lux at plant level. One lot of plants was grown outdoors from May to July 1983.

Embryo culture. Embryos were dissected aseptically, using tungsten knives (Cutter 1967), and placed immediately in a dish containing Monnier's embryo culture medium (Monnier 1976) to prevent desiccation. Mid-cotyledon- and maturation-stage embryos were selected on the basis of morphology. The mid-cotyledon stage corresponds to 30–35 d post-anthesis (dpa), 3.3–4.9 mg fresh weight (FW) per embryo, after cell division has ceased and storage proteins are accumulating rapidly; the maturation stage corresponds to 45–50 dpa, 5.5–6.9 mg FW per embryo, just after embryo water content starts to drop and storage-protein accumulation is slowing down (Ching et al. 1974; Crouch and Sussex 1981). The embryos were then transferred to basal medium for culture (described below). Mature dry seeds were harvested from fully desiccated pods of field-grown plants. The dry seeds were soaked in 95% ethanol to dissolve the waxy coats, surface-sterilized in commercial bleach diluted 1:4 (1% sodium hypochlorite) for 20 min, and rinsed several times with sterile water before removing the embryos for culture. Six embryos were placed in each baby-food jar (5.5 cm diameter, 7 cm tall). Germinating seedlings were harvested at one-week intervals, at which time the organs were dissected apart. Tissue for protein determinations was washed three times by filtration with deionized, glass-distilled water, blotted, weighed, and stored at −80° C. Tissue for RNA extractions was frozen immediately in liquid nitrogen and stored at −80° C.

The culture medium of Monnier (1976) contains inorganic salts, reduced nitrogen, 0.35 M sucrose (12%), and is hormone-free. For the basal medium the sucrose concentration was lowered to 0.03 M (1%). The media ingredients except agar were mixed, pH was adjusted to 5.5 with 1.0 N KOH, and powdered agar (Difco-Bacto; Difco Laboratories, Detroit, Mich., USA) was added to 0.7%. The medium was autoclaved and dispensed 30 ml per jar. The jars were sealed with plastic lids (Magenta Corp., Chicago, Ill., USA) and cultured at 24° C in continuous light from cool-white fluorescent lamps (General Electric), 1940–2370 lux at plant level.

Quantitation of storage protein. Antibodies raised against purified 12 S stororage protein, cruciferin, were used in rocket immunoelectrophoresis to quantitate cruciferin in crude extracts as described by Crouch and Sussex (1981). Samples were prepared by homogenizing plant tissue in a conical sintered-glass homogenizer (Duall, Kontes of Illinois, Evanston, Ill., USA) attached to a Tri-R stirrer (Tri-R Instruments, Rockville Centre, N.Y., USA), on ice. The final sample dilution was 4 ml buffer per 1 g FW. Extracts were frozen at −80° C for at least 1 h, thawed and cleared by centrifugation for 15 min at 12500 *g*. The samples were subjected to electrophoresis across an agarose (low-mr; Biorad Laboratories, Richmond, Cal., USA) gel containing anti-cruciferin antibodies at 100 V for at least 12 h. The gels were stained with Coomassie Blue R-250 (Biorad Laboratories) and rocket area was measured by weighing tracings. Known standards were included on each plate and used to convert rocket area to concentration of cruciferin storage protein. The limit of detection was 10 ng cruciferin mg^{-1} FW of tissue.

Extraction of RNA. Total cellular RNA from organs of mid-cotyledon-stage embryos was prepared by phenol extraction as described in detail by Galau et al. (1981). Briefly, the plant tissue was homogenized on ice, in a conical sintered-glass homogenizer in 20 ml of homogenization buffer per 1 g FW of tissue. The homogenate was extracted with phenol-chloroform and the aqueous phase was precipitated twice in 100% ethanol, followed by precipitation with an equal volume of 4 M lithium chloride and a final ethanol precipitation. The RNA was stored in TE solution (10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol(Tris)-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6) at −80° C.

To avoid problems of polysaccharide contamination, RNA was prepared from the other samples (all tissues from maturation and dry-seed embryos, as well as primary cotyledons of mid-cotyledon-stage embroys three and four weeks after germination) by CTAB (cetyltrimethylammonium bromide) extraction as described below, based on the method of Taylor and Powell (1982). The yield and quality of RNA prepared by this technique was equivalent to that prepared by phenol extraction, but without polysaccharide contamination. Plant tissue was homogenized in a conical sintered-glass homogenizer in 2 ml 2× extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) plus 2% β-mercaptoethanol per 1 g FW, then transferred to tubes placed in a water bath set at 50–55° C, and allowed to equilibrate. Samples were extracted twice with an equal volume of SEVAG (chloroform:isoamyl

alcohol, 24:1 v/v). The nucleic acids were precipitated with an equal volume of precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% β-mercaptoethanol, added just before use), allowing 1 h at room temperature for complete precipitation. The precipitate was pelleted at 4000 *g* for 5 min, drained, and resuspended in 1 M NaCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA and a drop of β-mercaptoethanol, added just before used. Samples were ethanol-precipitated and resuspended in TE plus a drop of B-mercaptoethanol. The DNA was removed by LiCl precipitation overnight at −20° C. The RNA-containing pellet was resuspended in TESS (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 100 mM NaCl) and a drop of β-mercaptoethanol, then ethanol-precipitated. The pellets were washed in ethanol, vacuum-dried, and resuspended in TE.

Quantitation of RNA. Total RNA was measured by A 260 using the estimate that RNA at a concentration of 40 μg ml^{-1} absorbs one A unit at 260 nm. Values for total RNA were checked in two ways: 1) Samples of equal mass, based on A 260 were electrophoresed on 3-(N-morpholino)propanesulfonic acid-formaldehyde RNA gels (Bruskin et al. 1981). The gels were photographed and the negatives scanned with a densitometer. Estimates of sample concentrations were normalized according to the peak area of the rRNA bands. 2) Ribosomal-RNA (rRNA) levels in samples of equal mass, based on A 260, along with a dilution series of RNA of known concentration, were measured by dot blots (described below). The filter was probed with a nick-translated rDNA clone from *Raphanus sativus* L. (pRE12, a gift of Dr. M. Delseny, Université de Perpignan, France; described in Delseny et al. 1983) and the spots cut out and counted in a scintillation counter. Sample concentrations were determined relative to the standard curve.

After determining, by Northern blot analysis (Bruskin et al. 1981), that the cruciferin transcripts were the same size in all tissues, cruciferin mRNA levels were measured by dot blots (Thomas 1980). Nitrocellulose filters were presoaked in 20X SSC (0.15 M NaCl, 0.015 M sodium citrate), then dried. Samples of RNA of varying mass plus total yeast RNA (Sigma Chemical Co., St. Louis, Mo., USA; type XI) to make a total of 10 μg per spot were dried, resuspended in 3 μl TE per spot, boiled 2 min, and placed in ice immediately. Duplicate 3-μl spots of each sample were loaded in a grid on the filter. The filter was hybridized using the Northern blot conditions of Bruskin et al. (1981), probing with a nick-translated cruciferin complementary-DNA (cDNA) clone present in at least tenfold excess. Probes were prepared as described by Crouch et al. (1983) and had specific activities of $0.5 \cdot 10^8$–$1.0 \cdot 10^8$ cpm μg^{-1}. The spots were cut out, dissolved in 60% toluene, 40% methoxyethanol and 0.11% Omnifluor (New England Nuclear, Boston, Mass., USA), and counted in a scintillation counter. Cruciferin mRNA levels were determined relative to a standard curve of plasmid containing the cruciferin cDNA.

Results

Morphology. Embryos excised from mature dry seeds and placed on basal culture medium germinate rapidly. The radicle begins to elongate within 1 d, and hypocotyl elongation and cotyledon expansion begin within 2 d. Though the mature embryo does not have any well-developed leaf primordia at the shoot apex, the first true leaves form within a week of germination. Leaves can be distinguished from cotyledons on the basis of morphology: leaves are ovate with undulate margins and are slightly hirsute, cotyledons are obcordate and glabrous (Fig. 1 h).

When mid-cotyledon-stage embryos are excised and placed in culture, the cotyledons expand, the radicle elongates, and root hairs form within 2–3 d. However, subsequent development is unlike that of germinating mature embryos. The hypocotyls fail to elongate past about 5 mm in length, callus and secondary embryos are often formed on the hypocotyl, and no true leaves are formed. After a week in culture, secondary cotyledons begin to develop from primordia at the apex (Fig. 1 d, e). Since no primordia are visible at the apex at the time the embryos are placed in culture, the secondary cotyledons must form de novo. They are initiated sequentially and form a rosette, analogous to the ontogeny of true leaves on normal seedlings (compare Fig. 1 e and g). The secondary cotyledons remain in a tight rosette for at least four weeks. During the fourth week in culture the hypocotyls elongate and one to two weeks later the axis of the cotyledon rosette elongates (Fig. 1 f). Occasionally shoots with true leaves are formed, but always in cotyledon axils. In contrast, maturation-stage embryos cultured under these conditions germinate to form morphologically normal seedlings (Fig. 1 i).

Storage-protein synthesis. Levels of cruciferin in the various organs of normally and precociously germinating embryos were measured by rocket immunoelectrophoresis. Cruciferin-mRNA levels were measured by dot blots and standardized relative to total RNA in the sample. The accumulation pattern of both cruciferin protein and mRNA varied substantially among organs and among developmental stages, as detailed below.

Embryos from mature dry seeds degrade their storage proteins very rapidly during germination (Fig. 2 g, h). After only one week in culture, no immunocompetent material is present in any of the organs. Cruciferin-mRNA levels were measured only in ungerminated dry-seed embryos and 24-h imbibed embryos; in both cases no cruciferin mRNA was detected (data not shown).

In mid-cotyledon-stage embryos, the concentration of cruciferin in the original cotyledons decreases gradually, from an initial value of 2.7 μg mg^{-1} FW to 1.72 μg mg^{-1} FW after four weeks in culture (Fig. 2 a). In the hypocotyl, cruciferin levels decline over the first week, increase 2 to 3-fold over the second and third weeks to 5.72 μg mg^{-1} FW, and then decrease to 0.12 μg mg^{-1} FW when the hypocotyl elongates

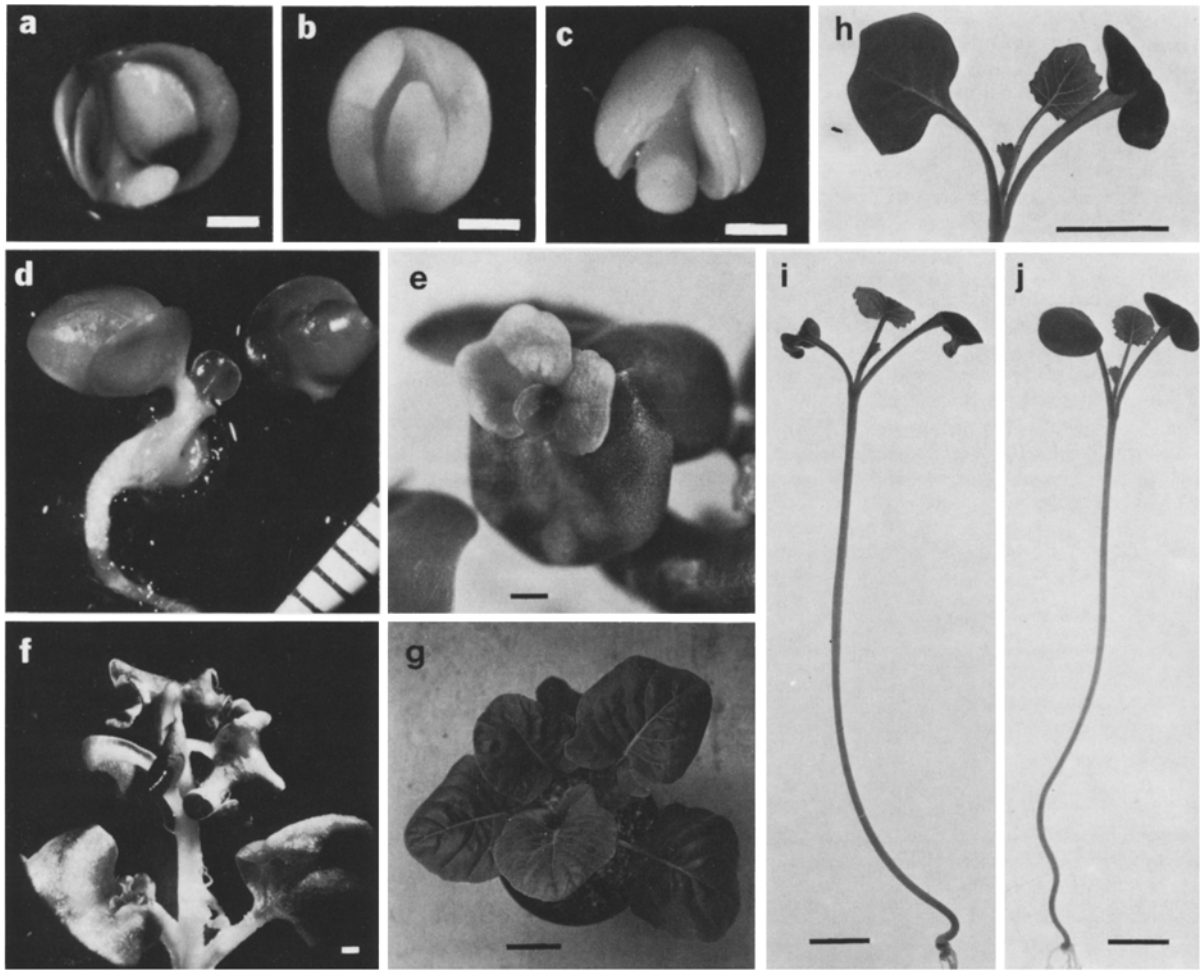

Fig. 1 a–j. Morphology of *B. napus* embryos, before and after germination. **a–c** Before germination: mid-cotyledon stage (**a**); maturation stage (**b**); mature dry (**c**). **d–f** Precociously germinating mid-cotyledon stage: after one week in culture, one cotyledon detached to expose secondary cotyledon at apex (**d**); after three weeks in culture (**e**); after four weeks in culture, apex with axis of cotyledon rosette elongating (**f**). **g** Phyllotaxy of *B. napus* plant. **h** Apex of 11-d seedling from dry-seed embryos. **i** Two-week seedling from maturation stage embryo. **j** 11-d seedling from dry-seed embryo. Magnification: **a** ×7, **b** ×8, **c** ×8.5, **d** ×4, **e** ×5, **f** ×2, **h** ×1.5. Scale bars: **a–f** = 1 mm, **g** = 5 cm, **h–j** = 1 cm

during the fourth week. The decreases in cruciferin concentration in the original organs may be a consequence of either breakdown or dilution (caused by the large increase in fresh weight of these tissues.) To distinguish between these possibilities, the values were converted to a per-organ basis (Fig. 2b). This approach indicated that the decrease in cruciferin concentration in the cotyledons was caused by dilution since cruciferin continued to accumulate in these organs. However, the decrease in cruciferin per hypocotyl during the fourth week reflects breakdown as well as dilution. The secondary cotyledons have low levels of cruciferin (0.29–0.37 μg mg^{-1} FW) during the first two weeks after they form, but the concentration increases tenfold during the third week, approaching that seen in the original cotyledons at the time of embryo excision. The timing of this increase in cruciferin concentration is also similar to that in the original cotyledons, where rapid accumulation begins the second week after they form. Surprisingly, the roots contained very low levels of cruciferin (0.02–0.05 μg mg^{-1} FW) throughout the culture period. These data indicate that excised mid-cotyledon-stage embryos retain embryonic characteristics at the biochemical level, producing embryo-specific proteins in both existent and newly formed embryonic organs and also in organs not normally present in embryos (e.g. roots).

The cruciferin-mRNA levels in the cotyledons of mid-cotyledon-stage embryos decrease fourfold over the first week in culture and then gradually decline to a low level that remains roughly constant through the culture period (Fig. 2c). In the hypocotyls, cruciferin-mRNA levels decrease gradually, showing little similarity to the dramatic fluctua-

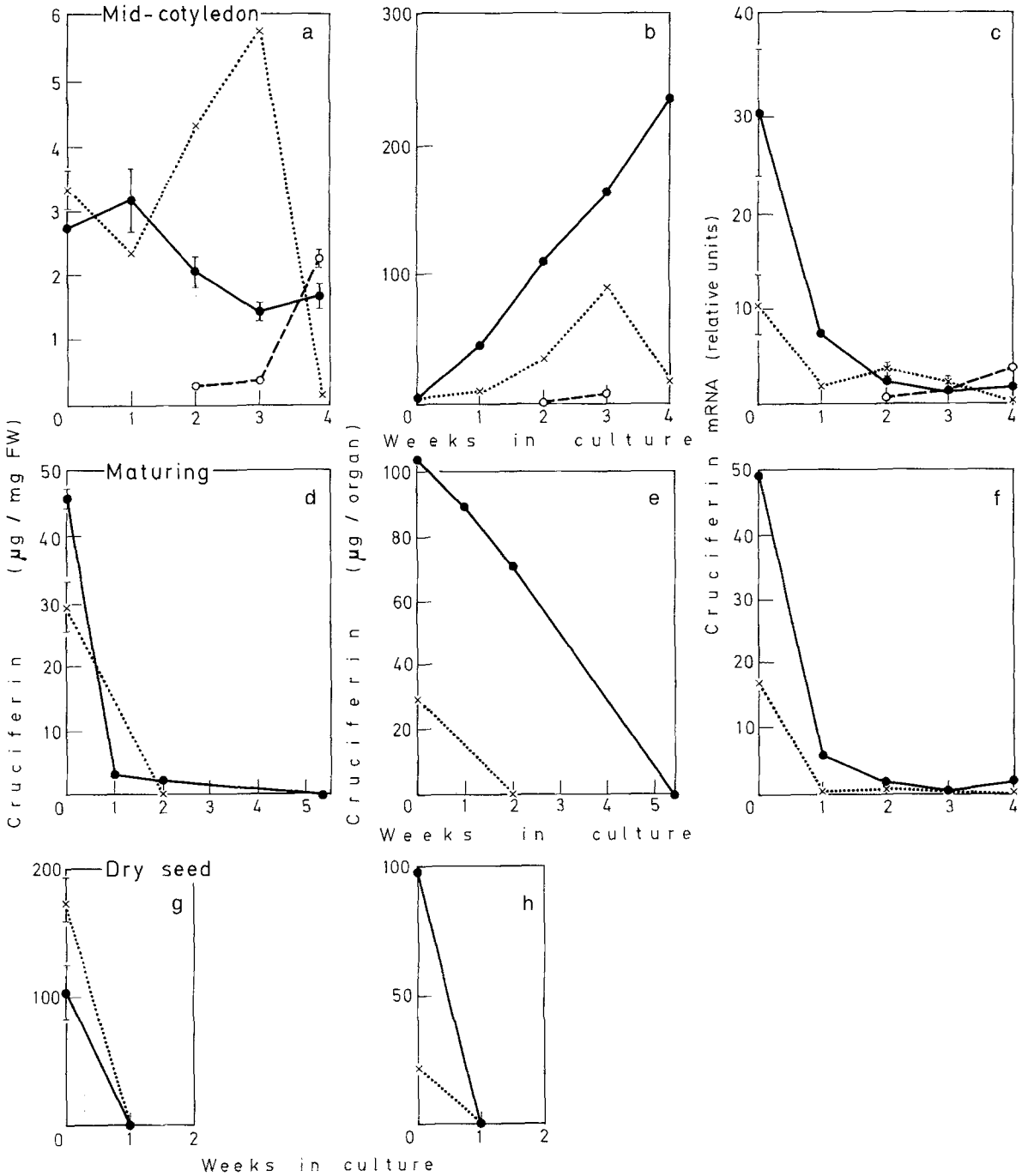

Fig. 2a–h. Levels of cruciferin protein and mRNA in organs of precociously and normally germinating *B. napus* embryos. Embryos were cultured at mid-cotyledon stage (**a–c**), maturation stage (**d–f**), or from dry seeds (**g, h**). Cruciferin concentrations (**a, d, g**) were determined by rocket immunoelectrophoresis. These values were converted to a per-organ basis (**b, e, h**) by multiplying by the average FW per organ. Cruciferin-mRNA levels were determined relative to total cellular RNA by the dot-blot procedure. Cruciferin protein and mRNA were detectable in roots and leaves of precociosuly germinating embryos, but the levels were too low to appear on these graphs. Each point represents the average of duplicate measurements from a single tissue sample derived from 20–40 embryos at younger stages and at least three seedlings at older stages. Error bars = SD. ● = Primary cotyledons; × = hypocotyls; o = secondary cotyledons

tions seen in protein concentration. Cruciferin-mRNA levels increase gradually in the secondary cotyledons, not reflecting the sharp increase in protein concentration during the fourth week. Although the cruciferin-mRNA levels in the secondary cotyledons at the end of the culture period are only about 10% of the initial levels in the original cotyledons, the protein concentration at this time is about 80% of that in the original cotyledons at the time of excision. The fact that relatively

low levels of cruciferin mRNA are sufficient for cruciferin accumulation indicates that this protein is very stable in these organs.

In precociously germinating maturation-stage embryos, the concentration of cruciferin in the cotyledons decreases rapidly, from 45.9 μg mg^{-1} FW to 2.1 μg mg^{-1} FW after only two weeks in culture (Fig. 2d). By five weeks in culture many of the cotyledons are senescing and those remaining have no detectable cruciferin left. The decrease in the hypocotyl is even more dramatic – by two weeks these organs are devoid of cruciferin. Very low levels of cruciferin appeared in roots and leaves, showing a transient accumulation in the leaves to 0.35 μg mg^{-1} FW after three weeks in culture and disappearing by five weeks. Measurements of cruciferin-mRNA levels roughly parallel the protein data (Fig. 2f). In cotyledons, both mRNA and protein levels drop 20 to 25-fold in the first two weeks of culture and then stabilize at a low level. The hypocotyl also contains low levels of cruciferin mRNA throughout the culture period. The continued presence of cruciferin mRNA in all organs of these seedlings presumably reflects continued transcription of the embryo-specific gene for cruciferin, indicating that they retain some embryonic characteristics. However, cruciferin is not stable in these seedlings, demonstrating that they have acquired the capacity to degrade it.

Discussion

Germination is frequently defined as radicle emergence, with subsequent growth classified as seedling development. The only signs of germination exhibited by mid-cotyledon-stage embryos were radicle extension and cotyledon expansion. Otherwise these embryos maintained an embryogenic pattern of development, forming extra embryonic structures (cotyledons) and continuing to accumulate storage protein in the original organs. However, unlike embryonic development in situ, the concentration of storage protein in the original cotyledons decreased becasue of large increases in fresh weight. The secondary cotyledons accumulated cruciferin to a concentration approaching that seen in the original cotyledons and over a comparable time-span, reflecting their embryonic character at the biochemical level. Fairly low levels of cruciferin mRNA were apparently sufficient for continued protein accumulation, indicating that the protein is very stable in these tissues. This is consistent with turnover studies demonstrating that storage proteins are very stable in isolated cotyledons of peas and soybeans (Madison et al. 1981). The only event characteristic of normal seedling development, elongation of the hypocotyl, was accompanied by degradation of cruciferin in the hypocotyl. Thus, the germination-specific process of storage-protein degradation occurred only in tissue which had ceased to develop embryonically at the morphological level as well.

Maturation-stage embryos exhibit an intermediate pattern of development. Morphologically, they appear to develop as normal seedlings, though at a slightly slower rate than those from mature dry seeds. At the biochemical level, measurements of cruciferin per organ indicate that these embryos degrade the storage proteins present in the original organs but at a much slower rate than germinating embryos from dry seeds. Low levels of cruciferin mRNA are present throughout the culture period and the extended period required for complete degradation may reflect a continued low-level synthesis of cruciferin which must then be degraded. In contrast, cruciferin-mRNA levels drop to undetectable levels in embryos which have developed to a comparable age in situ, i.e. embryos in dry seeds. The new structures formed in developing seedlings of maturation-stage embryos – roots and leaves – show transient very low level accumulations of cruciferin and cruciferin mRNA.

We conclude that, based on the criterion of root growth, developing *B. napus* embryos do germinate, but the capacity to develop as normal seedlings is acquired gradually during the maturation process. Thus, maturing rapeseed embryos, when germinating precociously, exhibit characteristics of both embryogeny and seedling development.

The synthesis of storage proteins in roots and leaves of precociously germinating, undesiccated *B. napus* embryos is unusual because storage proteins are restricted to embryonic structures during normal development (Crouch and Sussex 1981). In fact, in all species examined, storage-protein synthesis is under strict developmental control (for review, see Millerd 1975). This seed specificity appears to be the result of transcriptional regulation. For example, Goldberg et al. (1983) report that storage-protein transcription in soybeans is undetectable in various organs of mature plants, indicating that storage-protein genes are inactive in adult tissues. It is not known what signals termination of storage-protein transcription or when this occurs during normal embryo development. Our experiments indicate that, although dry seeds no longer contain storage-protein mRNA, termination has not occurred by the maturation stage. Furthermore, the accumulation of cruciferin mRNA and protein is truly stage-, not organ-, spe-

cific since the genes are active in organs which are not normal embryonic structures.

Although precocious germination has been observed in many other species, the continued embryo-like development of precociously germinating *B. napus* embryos appears to be unique. For example, studies with embryos of fruit trees (Tukey 1938), wild rice (LaRue and Avery 1938), and *Phaseolus* (Walbot et al. 1972) demonstrated that very young embryos germinated slowly and formed stunted plantlets, presumably reflecting inadequate nutrition. However, they did not form extra embryonic structures, although they sometimes failed to produce as many leaves as a germinating mature embryo. Furthermore, embryos of a comparable stage to those used in the present study, i.e. having completed histodifferentiation and growing by cell enlargement only, always formed morphologically normal seedlings. Extra cotyledons sometimes form on somatic embryos (for example, caraway; Ammirato 1977), but these appear to initiate simultaneously and form a whorl, unlike those of *B. napus* which initiate sequentially. The fact that maturation-stage embryos of *B. napus* form morphologically normal seedlings while retaining some embryonic characteristics at the biochemical level indicates that normal morphology is not proof of "maturity". However, the few biochemical studies in other species indicate that germination processes begin and embryo-specific processes stop when seedlings develop precociously. In cotton, the germination-specific enzyme, carboxypeptidase C, appears within a day of precocious germination (Ihle and Dure 1969), and in *Phaseolus*, storage-protein synthesis ceases upon visible germination (Long et al. 1981). Thus the continued embryo-like development of *B. napus* during precocious germination is unusual and may reflect a requirement for some exogenous signal to complete the maturation process.

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