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Role of ABA in Maturation of Rapeseed Embryos[1]

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ABSTRACT

Development of *Brassica napus* L. cv Tower embryos of different ages cultured *in vitro* with and without abscisic acid (ABA) was compared with normal development *in situ* to investigate the role of ABA in embryo maturation. Endogenous ABA levels were measured by radioimmunoassay, and sensitivity to ABA was assayed in terms of its ability to suppress precocious germination and stimulate accumulation of storage protein and storage protein mRNA. During development *in situ*, the levels of endogenous ABA and 12S storage protein mRNA both reach their peaks just before the embryos begin to desiccate. The ABA levels during this phase of development also correlate with the time required in culture before germination is evident. Following these peaks, increasing concentrations of exogenous ABA are required to both suppress germination and continue storage protein accumulation *in vitro*. Thus, both endogenous ABA and ABA sensitivity decline during maturation. The concentrations of exogenous ABA required to suppress germination at these later stages result in abnormally high levels of endogenous ABA and appear to be toxic. These results are consistent with the hypothesis that in maturing rapeseeds, low water content rather than ABA prevents germination during the later stages of development.

Embryogenic development ceases during maturation, the final phase of seed development in most angiosperms. During this phase, the seeds desiccate and enter a period of developmental arrest which prevents them from germinating before environmental conditions favor seedling growth. Precocious germination of embryos during development in seeds would risk desiccation and therefore be lethal. Several lines of evidence suggest that ABA plays a role in suppression of precocious germination. Exogenous ABA can block both visible germination and the appearance of germination-specific enzymes in cultured embryos (16). Measurements of plant growth regulator levels have shown a correlation between seed dormancy and high endogenous ABA (reviewed in 26). Viviparous mutants show a decrease in either ABA levels or ABA sensitivity (17, 24). Finally, vivipary can be induced by treatments which inhibit ABA synthesis (11) or deplete endogenous ABA pools (1).

Our earlier studies with *Brassica napus* showed that ABA was required not only to inhibit precocious germination in culture, but also for the continued expression of embryo-specific storage protein genes (6). The embryos in those studies were morphologically complete and were just beginning to accumulate storage reserves. These results suggest that ABA is important in maintaining embryonic development during the storage reserve accumulation phase of embryogeny. It is not known whether ABA also plays a role in regulating the transition from embryogeny to the state of developmental arrest preceding germination which occurs as the embryos desiccate. Therefore, we determined whether cessation of embryogeny in maturing rapeseeds reflected changes in endogenous ABA levels, sensitivity to ABA, or both. ABA levels were measured by radioimmunoassay and sensitivity to ABA was assayed in terms of its ability to both suppress germination and stimulate accumulation of storage proteins and their mRNAs. Our results are consistent with the hypothesis that ABA is important in suppressing germination and maintaining embryogeny early in development, but not in desiccating embryos.

MATERIALS AND METHODS

Plants. Seeds of *Brassica napus* L. cv Tower (from Dr. W. D. Beversdorf, University of Guelph, Ontario) were planted in plastic flats in a 2:1:1 (by volume) mixture of soil, vermiculite, and perlite (Krum, Silbrico Corp., Hodgkins, IL) and grown in a constant environment room (13°C nights, 18°C days) for 2 weeks. The seedlings were then transplanted to 15.2-cm pots and grown to maturity. Light was supplied by a combination of fluorescent (cool-white, very high output, Sylvania, GTE Products Corp., Danvers, ME) and incandescent (40 w, Sylvania) lamps to give 16-h days. On the day of anthesis, flowers were pollinated and tagged with the date.

Embryo Culture. Embryos were dissected aseptically, using tungsten knives (8) and placed immediately either in liquid nitrogen or in a Petri dish containing Monnier's embryo culture medium (22) with appropriate modifications (described below). Embryos were selected on the basis of age (dpa[3]), and each stage was used for parallel measurements of endogenous ABA, storage protein, and storage protein mRNA levels. Mature dry seeds were harvested from fully desiccated pods of plants grown in a constant environment room. Dry seeds were soaked in 95% ethanol to dissolve the waxy coats, surface-sterilized in commercial bleach diluted 1:4 (1% v/v sodium hypochlorite) for 20 min, and rinsed several times with sterile H_2O before removing the embryos for culture. Ten to 15 embryos were placed in each Petri dish (6 × 2 cm). Duplicate plates constituted each sample. Embryos were harvested after 3 d of culture. Tissue for ABA and protein determinations was washed three times by filtration in 1% (w/v) sucrose, blotted, weighed, and stored at −70°C. Tissue for RNA extraction was washed, blotted, weighed, frozen in liquid nitrogen, and stored at −70°C.

The culture medium of Monnier (22) contains inorganic salts, reduced nitrogen, 0.35 M sucrose (12%, w/v), and is hormone-free. For the basal medium, the sucrose concentration was lowered to 0.03 M (1%, w/v). For the ABA-containing media, ABA (mixed isomers, grade IV, Sigma Chemical Co.), 10 mM in

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[3] Abbreviations: dpa, days post anthesis; FW, fresh weight.

DMSO (50% v/v), was added to the basal medium to give final concentrations of 1 and 10 μM. In all cases, the media ingredients were mixed, pH was adjusted to 5.5 with 1.0 N KOH, and powdered agar (Difco-Bacto; Difco Laboratories) was added to 0.7% (w/v). The medium was autoclaved and dispensed 10 ml/dish. The dishes were sealed with Parafilm (American Can Co.) and cultured at 28°C in continuous light from cool white fluorescent bulbs (General Electric).

Extraction of ABA. Crude extracts for use in radioimmunoassay were prepared as described below, based on the method of Weiler (30). A conical sintered-glass homogenizer (Duall, Kontes of Illinois, Evanston, IL) attached to a Tri-R stirrer (Tri-R Instruments, Rockville Centre, NY) was used to grind 50 to 150 mg of tissue in 2.5 ml of 90% (v/v) methanol containing 10 mg/L 2,6 di-*t*-butyl-4-methylphenol (BHT). Each sample was divided into two tubes, and 150 ng of ABA (mixed isomers, grade IV, Sigma) were added to one tube as an internal standard of recovery efficiency. Samples were stored in the dark at 4°C for 48 h, with intermittent shaking. Extracts were cleared by centrifugation for 3 min at 12,500 *g*, diluted 5-fold with H_2O, and immunoassayed within 2 d.

Radioimmunoassay. Endogenous ABA was measured by radioimmunoassay according to the procedure of Weiler (30) using rabbit anti-ABA-human serum albumin serum (Miles-Yeda Ltd., Naperville, IL). This serum does not distinguish between the (+) and (−) enantiomers of ABA, but is specific for the *cis*, *trans*-isomer. Although it cross-reacts with esterified forms of ABA, it does not react with a variety of ABA metabolites and derivatives. All incubation steps were carried out in the dark and pipetting was done in dim light. All samples were assayed at least in duplicate. Each assay tube (13 × 100 mm, glass) contained 0.1 ml of sample or standard, 0.5 ml of 0.2 M Na acetate (pH 4.0), 0.1 ml of 1% (w/v) BSA (fraction V, Sigma) as carrier protein, and 0.1 ml of 1 ng/ml [^{3}H]ABA (15–20 Ci/mmol, Amersham) as tracer. After mixing, 0.1 ml of diluted antiserum was added and the tubes mixed again. For the determination of nonspecific binding, water was used instead of antiserum. The tubes were incubated for 90 min at 4°C and then 1 ml of hot saturated ammonium sulfate was added to each. Precipitation was allowed to occur for 30 min at room temperature and, after centrifuging and decanting, the pellets were washed once with 1 ml of half-saturated ammonium sulfate and then recentrifuged. The pellets were resuspended in 0.15 ml of water, transferred to scintillation vials, mixed with 3 ml of Aquasol (New England Nuclear), and counted in a scintillation counter.

Quantitation of Storage Protein. Antibodies raised against purified 12S storage protein, cruciferin, were used in rocket immunoelectrophoresis to quantitate cruciferin in crude extracts as described by Crouch and Sussex (6). Plant tissues were homogenized on ice in conical sintered-glass homogenizers in 4 ml buffer (19.2 mM Tricine, 64.8 mM Tris, 0.28 mM Ca lactate, 2.48 mM NaN_3) per 1 g FW. Extracts were frozen at −70°C for at least 1 h, thawed, and cleared by centrifugation for 15 min at 12,500 *g*. The samples were subjected to electrophoresis across an agarose (low M_r; BioRad Laboratories) gel containing anticruciferin 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. Cruciferin standards were included on each plate and used to convert rocket areas to cruciferin concentration. The limit of detection was 10 ng cruciferin/mg FW of tissue.

RNA Extraction. Total cellular RNA was prepared by phenol extraction as described in detail by Galau *et al.* (12). Briefly, plant tissue was homogenized on ice in a conical sintered-glass homogenizer in 20 ml of homogenization buffer (0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.5% w/v SDS, pH 9) 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 LiCl and a final ethanol precipitation. The RNA was stored in 10 mM Tris-HCl, 1 mM EDTA (pH 7.6) at −70°C.

RNA Quantitation. Total RNA was measured by A_{260} using the estimate that RNA at a concentration of 40 μg/ml absorbs one *A* unit at 260 nm.

Since Northern blot analysis showed that the cruciferin and napin probes each hybridized to only one size class of transcript in all tissues, cruciferin and napin mRNA levels were measured by dot blots using the method of Galau and Thomas (personal communication). RNA was denatured in 50% (v/v) formamide, 6% (v/v) formaldehyde, 20 mM Na phosphate buffer (1:9, mono to dibasic) at 65°C for 5 min, then cooled on ice. After denaturing, the sample was diluted 1:10 to reduce the formamide concentration, but formaldehyde was kept at 6% to keep samples fully denatured. Serial dilutions of sample RNAs were made using similarly denatured yeast RNA as the diluent. No more than 2 μg of total RNA were loaded per spot and all samples were loaded in a volume of 150 μl. Nitrocellulose filters were presoaked in 10X SSC (75 mM NaCl, 7.5 mM sodium citrate) at least 1 h, then placed over 3MM filter paper on a dot blot manifold (Schleicher and Schuell, Keene, NH) and clamped securely. A slight vacuum was applied with an aspirator and duplicate spots or serial dilutions of each sample were loaded on the filter. After vacuum-baking, the filter was hybridized using the Northern blot conditions of Bruskin *et al.* (4), probing with ^{32}P-labeled nick-translated cruciferin or napin cDNA clones present in at least 10-fold sequence excess. Probes were prepared as described (7) and had specific activities of 0.5 to 1.0 × 10^8 cpm/μg. The spots were cut out, dissolved in 60% (v/v) toluene, 40% (v/v) methoxyethanol, and 0.11% (w/v) Omnifluor (New England Nuclear), and counted in a scintillation counter. Cruciferin and napin mRNA levels were determined relative to a standard curve created by serially diluting one of the RNA samples.

RESULTS

Endogenous ABA during Embryogeny. To determine whether the cessation of embryogeny in maturing rapeseed embryos reflected changes in endogenous ABA levels, ABA was measured by radioimmunoassay (Fig. 1). The serum used was specific for the *cis*, *trans*-isomer, and values obtained by this method were corroborated by GC-MS analysis of parallel samples for two stages. Endogenous ABA levels rise 3- to 4-fold during the storage protein accumulation phase (25–39 dpa), reaching peaks at 35 and 38 dpa. The second peak occurs just before embryo water

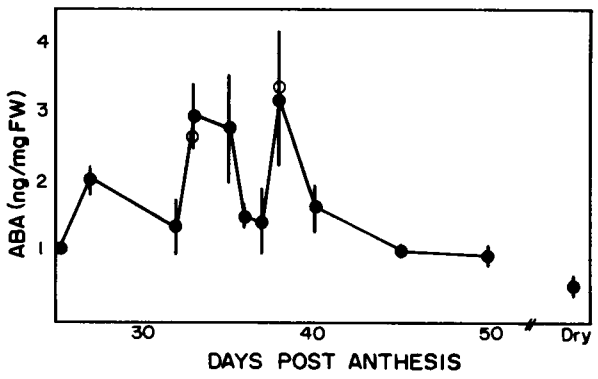

FIG. 1. Endogenous ABA during embryogeny. ABA was measured by radioimmunoassay. Each point represents the average of duplicate assays of at least two samples, each derived from 10 to 20 embryos. (○), values obtained by GC-MS. Error bars = SD. For comparison, endogenous ABA in subtending leaves quickly killed in liquid N_2 was 0.33 ng/mg FW.

content (mg H_2O/embryo) starts to drop (6) and is slightly higher, reaching about 3.3 ng/mg FW. Following this second peak, endogenous ABA declines rapidly to the prepeak level and remains at this level until seed maturity. Embryo ABA levels are quite high throughout the period; even the lowest levels are 3- to 4-fold higher than those in subtending leaves (0.3 ng/mg FW).

Storage Protein mRNA Levels during Embryogeny. Since exogenous ABA stimulates synthesis of storage proteins in cultured immature embryos, we were interested in comparing the time course of storage protein mRNA accumulation with endogenous ABA levels during embryo development in seeds. Total RNA was extracted from embryos of various ages, and relative mRNA levels for the two major classes of rapeseed storage proteins, cruciferin and napin, were measured by dot blots. Representative data are shown in Figure 2. The assay was nearly linear over a 250-fold range of RNA sequence concentration (Fig. 2c).

Figure 3 shows the relationship between embryo age and storage protein mRNA levels. Napin mRNA can first be detected at 18 dpa; the levels peak by 27 dpa and remain high until 40 dpa. Cruciferin mRNA is detected at 21 dpa and levels increase 3- to 4-fold from 27 dpa to 40 dpa, when they reach their peak. As the embryos desiccate, both cruciferin and napin mRNAs decrease rapidly to either very low or undetectable levels in dry seeds, depending on the lot of seeds. Although both transcripts are embryo-specific, the difference in timing of their expression indicates that their regulation is not tightly coordinated. It is interesting that cruciferin mRNA levels generally parallel changes in endogenous ABA levels (see Fig. 1).

Germination Capacity during Embryogeny. Since ABA has been implicated in suppressing germination of many species, we determined whether the length of time required for embryos to germinate reflected the endogenous ABA levels. Embryos of various ages were cultured on basal medium and scored for germination, using appearance of root hairs as a marker. The lag period to germination followed the same time course as endogenous ABA, with both the longest lags and highest ABA levels at 34 and 38 dpa (Fig. 4). These results are consistent with a role for ABA in suppressing germination during the storage protein accumulation phase of embryogeny.

Effects of Exogenous ABA on Cultured Embryos. To determine whether the embryos remain sensitive to ABA throughout maturation, embryos from 27 dpa (early cotyledon stage) to those from mature dry seeds were dissected from seeds and cultured for 3 d on basal medium with 10 μM ABA, 1 μM ABA, or no ABA. On hormone-free medium, embryos which did not have high endogenous ABA levels at the time of excision germinated within 2 to 3 d of culture (Figs. 4 and 5). Germination was inhibited by 1 μM ABA in embryos up to 40 dpa, but not in older embryos. Application of 10 μM ABA inhibited germination at all stages but was toxic in embryos older than 40 dpa. These results indicate that maturing embryos require increasing concentrations of exogenous ABA to suppress germination.

Endogenous ABA in Cultured Embryos. Since the embryos' response to exogenous ABA depended on their developmental stage, we asked whether endogenous ABA levels reflected the concentration of applied ABA. To do this, we measured endogenous ABA in embryos of various ages cultured as described above (Table I). In all stages cultured on basal medium, endogenous ABA drops to 0.3 ng/mg FW. In stages flanking the peaks, culture on 1 μM ABA maintains endogenous ABA at comparable levels to embryos developing *in situ*, while culture on 10 μM ABA raises endogenous ABA to abnormally high levels. Stages within or between the ABA peaks can be maintained at or raised to peak levels by application of 10 μM ABA, but 1 μM ABA is not sufficient. These data show that, at all stages, endogenous ABA levels show a dose response to exogenous ABA. To determine whether the endogenous ABA levels were a response to the exogenous levels or simply equilibration, they were converted to a μM basis. This approach indicated that the embryos were not merely equilibrating since endogenous levels were always higher than those applied. It is interesting that endogenous levels which are found *in situ* at younger stages are higher than those which are toxic at later stages. The fact that 45 dpa embryos cultured

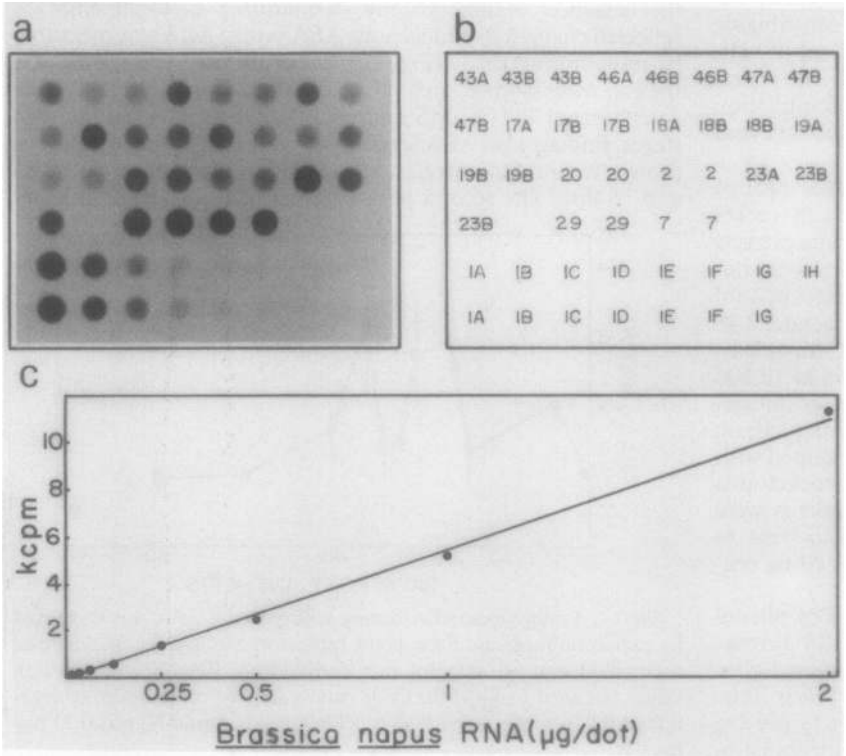

FIG. 2. Quantitation of storage protein mRNA. Samples of denatured total embryo RNA were spotted on nitrocellulose filters. Replicate filters were hybridized with nick-translated cDNA probes to mRNAs for rapeseed storage proteins. One of the samples was used to prepare a 2-fold dilution series of embryo RNA. Relative mRNA abundance was determined by scintillation counting of spots. a, Autoradiogram of dot blot; b, key to samples on dot blot: numbers refer to different RNA samples, A = 2-μg spot, B = 1-μg spot, etc.; c, standard curve.

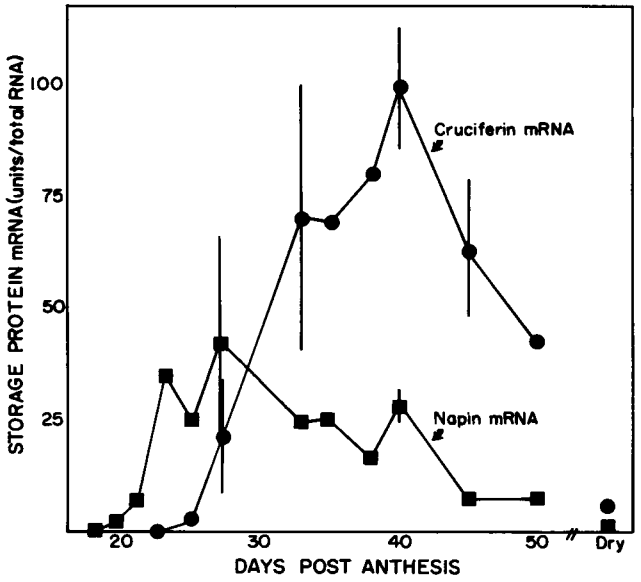

FIG. 3. Storage protein mRNA levels during embryogeny. Total RNA was extracted from embryos at the indicated days after fertilization. Relative levels of cruciferin (●) and napin (■) mRNA during embryogeny were measured by dot blots as described in Figure 2. Each spot represents the average of duplicate samples, each derived from 10 to 30 embryos. For embryos younger than 25 dpa, samples were derived from 10 mg of tissue. Error bars = SD.

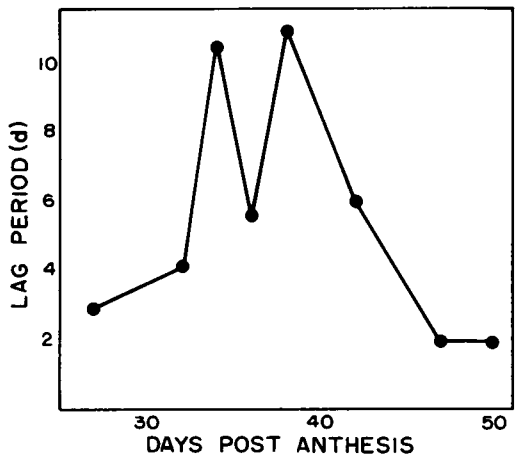

FIG. 4. Germination capacity during embryogeny. Embryos were cultured on hormone-free medium and scored daily for appearance of root hairs. Germination percentage was plotted *versus* time, and the number of d to germination of 95% of the embryos were determined from these curves. Each point represents samples of 50 to 200 embryos.

on 1 μM ABA germinate, although their endogenous ABA levels are the same as ungerminated embryos still in the seed, suggests that maturing embryos are losing sensitivity to ABA.

Storage Protein Synthesis in Cultured Embryos. Since ABA stimulates cruciferin synthesis in young embryos (6), and changes in the levels of ABA and cruciferin mRNA correlate well, sensitivity to ABA was assayed biochemically in terms of its effects on accumulation of cruciferin and its mRNA. Cruciferin levels in cultured embryos were measured by rocket immunoelectrophoresis. Levels of cruciferin mRNA were measured by dot blots and standardized relative to total RNA in the sample.

Both cruciferin and cruciferin mRNA levels show a dose response with respect to exogenous (and endogenous) ABA in embryos excised up to 40 dpa. However, while embryos cultured

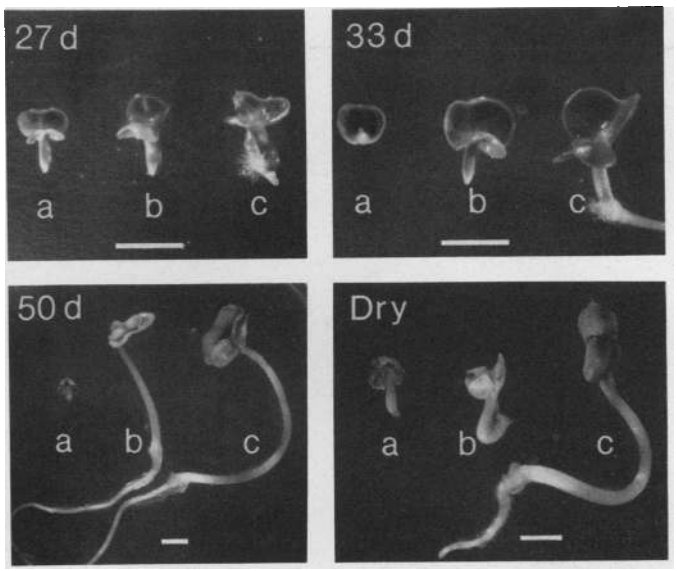

FIG. 5. Effects of exogenous ABA on cultured embryos. Embryos of the indicated ages were cultured for 3 d on basal medium with: a, 10 μM ABA; b, 1 μM ABA; or c, no ABA. Bar = 4 mm.

on 1 μM ABA accumulate cruciferin at the same rate as embryos developing in seeds (Fig. 6), the cruciferin mRNA levels in these embryos are lower than in embryos developing *in situ* (Fig. 7a). Since the concentration of total RNA is lower in cultured embryos than in those developing *in situ*, the difference in cruciferin mRNA levels is even greater when expressed relative to FW (Fig. 7b). The fact that relatively low levels of cruciferin mRNA are sufficient for rapid cruciferin accumulation suggests that this protein is very stable in embryos younger than 40 dpa. This is supported by the fact that cruciferin per embryo increases in predesiccation stage embryos under all culture conditions (data not shown). Another possibility is that ABA enhances translation of cruciferin since less cruciferin mRNA, but more cruciferin protein, accumulates per embryo at increasing ABA concentrations (data not shown). In cultured embryos excised after 40 dpa, the dose response is no longer apparent when cruciferin mRNA levels are expressed relative to total RNA, but it is still seen for both cruciferin and its mRNA when their concentrations are expressed relative to FW. However, neither 1 nor 10 μM ABA was sufficient to maintain cruciferin accumulation in these embryos at the same rate as in embryos developing in seeds. The cruciferin concentration actually decreased in desiccation-stage embryos cultured on 1 μM or no ABA, presumably reflecting degradation or dilution as the embryos germinated. These data indicate that maturing embryos lose sensitivity to ABA with respect to its ability to maintain embryogeny, as assayed by cruciferin synthesis.

Synthesis of the other major rapeseed storage protein, napin, is also enhanced by ABA. Although we have no convenient assay for napin protein, we did measure napin mRNA levels in cultured embryos (Fig. 8). Once again, young embryos showed a dose response but the levels were much lower than in embryos developing *in situ*. The embryos appear to lose sensitivity to ABA around 40 dpa, but it is not clear why the dose response reappears at 45 dpa.

DISCUSSION

Many studies have implicated abscisic acid in maintaining embryonic development and suppressing germination. Analysis of viviparous mutants of corn (3, 24) and *Arabidopsis* (17, 19) have shown that precocious germination of these embryos is

Table I. *Endogenous ABA in Cultured Embryos*

ABA (ng/mg FW) was measured by radioimmunoassay in embryos of various ages cultured for 1 or 3 d on basal medium with 10 μM ABA, 1 μM ABA, or no ABA. Values were converted to a μM basis, indicated in parentheses, making the assumption that all water in the tissue was available to the ABA. Water content (mg H_2O/mg FW) was determined for embryos samples cultured in parallel with those used in ABA extractions. Values represent the average of duplicate assays of at least two samples, each derived from 10 to 20 embryos.

Stage	Endogenous ABA			
	Upon excision	Cultured 3 d with		
		No ABA	1 μM ABA	10 μM ABA
		ng/mg FW (μM)		
25 dpa	1.05 ± 0.05 (5.18)	0.29 ± 0.06 (1.35)	1.49 ± 0.51 (8.2)	7.35 ± 0.01 (38.1)
32 dpa	1.39 ± 0.40 (7.74)	0.30 ± 0.2 (1.55)	0.93 ± 0.2 (5.38)	2.11 ± 0.6 (13.7)
37 dpa	1.49 ± 0.48 (9.97)	0.23 ± 0.15 (1.25)	1.42 ± 0.89 (9.28)	6.1 ± 1.2 (43.3)
38 dpa	3.28 ± 0.95 (20.8)	0.33 ± 0.16 (1.75)	0.85 ± 0.76 (5.2)	3.43 ± 0.8 (16.6)
45 dpa	1.07 ± 0.16 (8.7)	0.29 ± 0.01 (1.26)	1.21 ± 0.56 (5.9)	3.55 ± 1.22 (26.5)
Dry	0.54 ± 0.15 (29.7)	0.28 ± 0.11 (1.22)	0.61 ± 0.06 (2.74)	1.3 ± 0.11 (8.84)

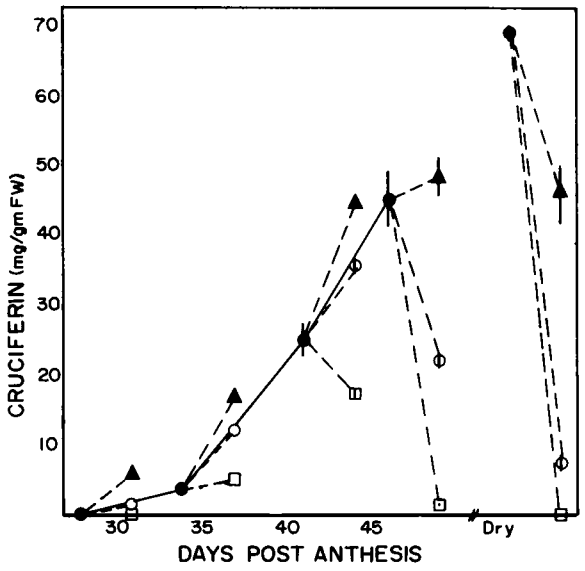

FIG. 6. Cruciferin accumulation in cultured embryos. Embryos of various ages were cultured for 3 d on basal medium with 10 μM ABA (▲), 1 μM ABA (○), or no ABA (□). Cruciferin concentrations were determined by rocket immunoelectrophoresis. The solid lines connect the levels found in embryos developing *in situ*. The dashed lines indicate the changes occurring during the culture period. Each point represents the average of duplicate measurements from a single tissue sample derived from 10 embryos. Error bars = SD.

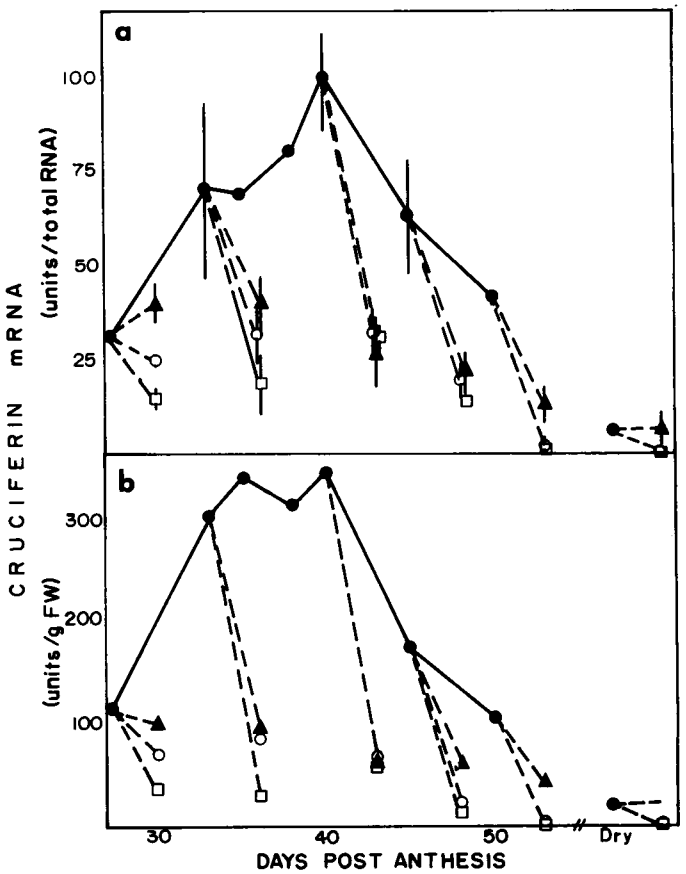

FIG. 7. Cruciferin mRNA in cultured embryos. Embryos of various ages were cultured for 3 d on basal medium with 10 μM ABA (▲), 1 μM ABA (○), or no ABA (□). Cruciferin mRNA levels were determined relative to total cellular RNA (a) by the dot blot procedure. The cruciferin levels were converted to a per-FW basis (b) by multiplying by the average mg RNA/g FW. (——), connect the levels found in embryos developing in seeds. (---), changes occurring during the culture period. Error bars = SD.

associated with a decrease in either endogenous ABA levels or ABA sensitivity. Fluridone treatment to inhibit ABA synthesis in developing corn results in vivipary (11) and treatments which deplete endogenous ABA pools can induce precocious germination in soybeans (1). Experiments tracing the appearance of biochemical markers in embryos cultured with or without ABA have shown that ABA inhibits synthesis of germination-specific enzymes such as isocitrate lyase and proteases in cotton (16) and α-amylase in barley (14), but stimulates synthesis of embryo-specific proteins in rapeseed (6), rice (25), and wheat (28). Furthermore, ABA appears to play a role in the maturation of

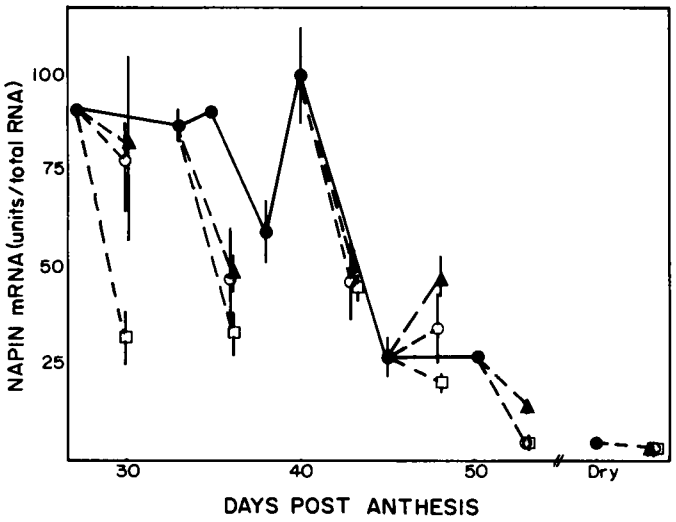

FIG. 8. Napin mRNA in cultured embryos. Embryos of various ages were cultured for 3 d on basal medium with 10 μM ABA (▲), 1 μM ABA (○), or no ABA (□). Napin mRNA levels were determined relative to total cellular RNA by the dot blot procedure. The solid lines connect the levels found in embryos developing in seeds. The dashed lines indicate the changes occurring during the culture period. Error bars = SD.

cotton embryos since young embryos cultured on ABA acquire a subset of proteins including a battery of enzymes normally appearing late in embryogeny (5, 9).

Exogenous ABA maintains embryogeny in cultured young rapeseed embryos, as reflected by increased cruciferin synthesis (6). To determine whether the effects ascribed to exogenous ABA could be mediated by endogenous ABA during normal development, we measured levels of endogenous ABA and storage protein mRNA in embryos developing in seeds. Although the antiserum used does not distinguish between the (+) and (−) enantiomers of ABA, both of these forms are highly active in inhibiting growth of wheat embryos and bean axes and blocking gibberellic acid induction of α-amylase production in barley half-seeds (reviewed in 29). Therefore, they are both presumably significant in embryonic development. We found that the highest levels of ABA are present just before cruciferin mRNA levels peak, consistent with a role for ABA in stimulating expression of these embryo-specific genes. Although napin mRNA is also an embryo-specific transcript whose accumulation is enhanced by ABA, it peaks much earlier and thus does not appear to be regulated in the same manner as cruciferin. The strong correlation between endogenous ABA at the time of excision and lag period to germination in culture suggests that ABA suppresses germination during the storage protein accumulation phase of embryo development. However, endogenous ABA decreases rapidly upon culture without exogenous ABA, indicating that ABA itself is not responsible for the failure to germinate throughout the lag period. The fact that ABA levels decline as the embryos lose water and enter a period of developmental arrest suggests that ABA is not directly involved, if at all, in blocking germination during the desiccation phase. Our data identified two peaks in ABA levels, occurring at 35 and 38 dpa. It was impossible to determine highly accurate values between 35 and 38 dpa because the levels are changing dramatically and, even with tagged siliques from hand-pollinated flowers, embryo age may vary as much as two to three d within a given silique. This two-peaked pattern of embryonic ABA levels is similar to that reported in *Phaseolus* (15), wheat (18), barley (23), and *Arabidopsis* (17). In the case of *Arabidopsis*, genetic analysis has identified the early peak as being of maternal origin, while the second peak is embryonic and is responsible for induction of dormancy. Exogenous ABA behaves as maternal ABA in that it cannot suppress germination of embryos lacking embryonic ABA. Our experiments do not allow us to identify the source of the ABA peaks in *Brassica*, but both are correlated with the suppression of germination.

Although the levels of ABA decrease during embryo maturation, ABA might still be suppressing germination if these embryos have become hypersensitive to ABA as suggested by Trewavas' (27) tissue sensitivity model of hormone action. To test this hypothesis, we measured ABA sensitivity in terms of the response to exogenous ABA of embryos cultured at various stages. At all stages, endogenous ABA levels in the cultured embryos reflected the concentration of applied ABA. However, it was not clear from these experiments whether the applied ABA was being taken up against a concentration gradient, as has been demonstrated in cotton embryos (13), or was inducing embryonic synthesis of ABA. Applied ABA inhibits germination in embryos which have not yet begun to lose water (on a mg water/embryo basis; water concentration is decreasing throughout embryo development [6]), but desiccating embryos require higher levels of ABA to suppress germination. Thus, the older embryos appear to be less sensitive to ABA. Furthermore, necrosis of these older embryos when exposed to high ABA suggests that the growth inhibition is an effect of ABA toxicity. The fact that desiccating embryos germinate in culture even under conditions that maintain endogenous ABA at physiological levels shows that these ABA levels are not sufficient to block germination at this stage of development.

At the biochemical level, ABA sensitivity was assayed in terms of its effect on synthesis of the storage protein cruciferin. Culture on ABA stimulated cruciferin accumulation in embryos excised prior to the desiccation phase, but none of the concentrations tried could sustain cruciferin accumulation in older embryos. To determine the level at which ABA affects cruciferin synthesis, we measured cruciferin mRNA levels. When expressed relative to total RNA, cruciferin mRNA also showed a dose response to ABA in embryos which had not begun to desiccate, but not in older embryos. Thus, the loss of ABA sensitivity is seen again, reflected in its inability to stimulate accumulation of cruciferin and its mRNA. The fact that ABA has a greater effect on accumulation of cruciferin than its mRNA suggests that ABA may be affecting storage protein synthesis at the level of protein stability or translation as well.

The present experiments indicate that an embryo's response to ABA is determined by its developmental stage. Our previous studies with rapeseed have identified phases of the maturation process in terms of the embryo's ability to germinate normally (10). Although embryos allowed to germinate precociously prior to the desiccation phase do show root growth, they retain many embryonic characteristics, both morphologically and biochemically. Embryos which have begun to lose water (mg H_2O/embryo) germinate to form morphologically normal seedlings, but continue to make the embryo-specific protein cruciferin. The timing of the onset of water loss correlates well with the transition out of the 'continued embryogeny' exhibited by immature embryos, consistent with a role for desiccation in the maturation process.

Desiccation, the final phase of normal embryonic development in most angiosperms, appears to be important in the transition from embryogeny to the ability to germinate and form normal seedlings. For example, many studies have demonstrated the importance of drying for subsequent germination of cereals (reviewed in 21). Armstrong *et al.* (2) found that the aleurone layer of wheat would not produce α-amylase in response to GA unless it was first sensitized to GA by drying to a critical water content. Similarly, premature drying of *Phaseolus vulgaris* and castor bean seeds can alter their developmental potential such

that, upon rehydration, they germinate rather than resuming embryogeny (20). Germination is reflected at the biochemical level by a change in the pattern of protein synthesis such that embryo-specific products disappear and germination-specific proteins appear. Thus, in several species, drying appears to act as a switch that turns off embryo-specific genes and allows germination-specific genes to become active. We are presently testing the effects of desiccation in *Brassica*.

In summary, ABA appears to be important in maintaining embryogeny and suppressing germination during the phase of rapid embryo growth preceding desiccation. However, the decline in both endogenous ABA and sensitivity to ABA during desiccation are consistent with the hypothesis that maturing rapeseeds rely on low water content or some factor other than ABA to suppress germination and complete the maturation process during the later stages of development.

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