Plant Physiol. (1986) 81, 907–912
0032-0889/86/81/0907/06/$01.00/0

Rapeseed Embryo Development in Culture on High Osmoticum Is Similar to That in Seeds[1]

Received for publication November 11, 1985

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ABSTRACT

The development of *Brassica napus* L. cv Tower embryos of different ages cultured *in vitro* with and without high osmoticum (0.48 and 0.69 molar sorbitol) was compared with normal development *in situ* to investigate the role of a drying environment in embryo maturation. Sensitivity to osmoticum was assayed in terms of its ability to mimic normal development, *i.e.* to both suppress germination and maintain 12 S storage protein (cruciferin) synthesis at levels comparable to those seen in the developing seed. The osmotic conditions used block germination of predesiccation stage embryos but were not sufficient to prevent desiccation stage embryos from taking up water and germinating. At all stages tested, the osmotically treated embryos had approximately normal levels of cruciferin mRNA. Measurements of endogenous abscisic acid (ABA) levels by radioimmunoassay indicated that the osmotic effects on germination and gene expression were not mediated by elevated embryonic ABA. Comparison of the kinetics of osmotic and ABA effects on gene expression showed that the osmotic effect is more rapid. These results are consistent with the hypothesis that ABA acts by inhibiting water uptake, which mechanically prevents germination and affects gene expression in some unknown manner.

At maturity, seed moisture content ranges from 5 to 15% water, depending on the species and the environmental conditions (reviewed by Adams and Rinne [1]). In contrast, young seeds in which the embryos have not yet begun to accumulate reserves have a much higher water content, between 80 and 90%. As the seeds accumulate reserves, their dry weight increases faster than their fresh weight, resulting in a decrease in percent water (23). During the final phase of seed development, desiccation, fresh weight decreases and the embryos enter a period of developmental arrest. Thus, the maturing seed is a drying environment and water loss is a normal part of embryo development, not a stress situation.

In many species, immature embryos can bypass the desiccation phase of development to germinate precociously when excised from seeds. However, the seedlings produced by precocious germination of immature embryos may be abnormal (7). Studies with both monocot and dicot embryos have indicated that desiccation is an important part of the maturation process. While precociously germinating rapeseed embryos exhibit a mixture of embryonic and germinative processes (7), premature drying and subsequent rehydration of immature embryos of wheat (16) and *Phaseolus vulgaris* (15) results in a shift in developmental potential from embryonic to germinative growth.

Our earlier studies with *Brasicca napus* showed that either ABA or high osmoticum could inhibit germination and stimulate synthesis of embryo-specific storage proteins in embryos cultured at early cotyledon stage, when they are just beginning to accumulate storage reserves, effectively mimicking the seed environment (4). We have since extended this work to later stages of embryo development to determine whether the embryos remain sensitive to these cues throughout embryogeny. Culture studies with exogenous ABA suggest that ABA is important in preventing germination and maintaining embryogeny only in predesiccation stage embryos (8). The present studies indicate that high osmoticum approximates the seed environment more closely than treatment with ABA throughout embryo development with respect to regulating these processes. Because osmotic stress has been shown to cause increased ABA levels in many plant tissues, we wanted to know if the effects of high osmoticum were mediated by endogenous ABA adjusted to appropriate levels by the embryos. Our results show that both inhibition of germination and maintenance of storage protein synthesis can be uncoupled from high ABA levels, suggesting that ABA is probably not the primary effector regulating these processes.

MATERIALS 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 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) and incandescent (40W, Sylvania, Seneca Falls, NY) lamps to give 16-h d. On the d of anthesis flowers were pollinated and tagged.

Embryo Culture. Embryos were dissected aseptically, using tungsten knives (6) and placed immediately either in liquid N_2 or in a Petri dish containing Monnier's embryo culture medium (17) with appropriate modifications (described below). Embryos were selected on the basis of age (dpa)[2] and pooled embryos of each stage were 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 NaOCl) 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

[1] Supported by grant PCM-83-16403 from the National Science Foundation to M.L.C.

[2] Abbreviation: dpa, days post anthesis.

for RNA extraction was washed, blotted, weighed, frozen in liquid N_2 and stored at −70°C.

The culture medium of Monnier (17) 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). Sorbitol was added as osmoticum to give final concentrations of 8.7, 1.9, or 12.5%. For the ABA-containing media, ABA (mixed isomers, grade IV, Sigma Chemical Co.), 10 mM in 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) was added to 0.7% (w/v). The medium was autoclaved and dispensed 10 ml per 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 (24). 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 ml of 90% (v/v) methanol containing 10 mg/L 2,6 di-t-butyl-4-methylphenol (BHT). Each sample was divided into 2 tubes and 100 ng of ABA (mixed isomers, grade IV, Sigma) was 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 12500*g*, diluted 5-fold with water, and immunoassayed within 2 d.

Radioimmunoassay. Endogenous ABA was measured by radioimmunoassay according to the procedure of Weiler (24) 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. In other studies, values obtained by this assay were corroborated by GC-MS (8). 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 sodium 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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ and then recentrifuged. The pellets were resuspended in 0.15 ml water, transferred to scintillation vials, mixed with 3 ml Aquasol (New England Nuclear), and counted in a scintillation counter.

Quantitation of Storage Protein. Antibodies raised against purified 12 S storage protein, cruciferin, were used in rocket immunoelectrophoresis to quantitate cruciferin in crude extracts as described by Crouch and Sussex (4). 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 fresh weight. 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 fresh weight of tissue.

RNA Extraction. Total cellular RNA was prepared by phenol extraction as described in detail by Galau *et al.* (9). 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 fresh weight 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 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.

Because Northern blot analysis showed that the cruciferin probe hybridized to only one size class of transcript in all tissues, cruciferin mRNA levels were measured by dot blots using the method of Galau and Thomas (personal communication), as previously described (10).

RESULTS

Effect of Osmoticum on Germination. Because the osmotic potential of the embryo decreases throughout seed development (*e.g. Phaseolus vulgaris* [25]), we wanted to know if high osmoticum could maintain embryonic development in cultured embryos of various stages. Embryos from early cotyledon stage (27 dpa) to those from mature dry seeds (60–70 dpa) were dissected from seeds and cultured for 3 d on basal medium supplemented with 12.5% (0.69 M), 8.7% (0.48 M), or no sorbitol. These concentrations were chosen because they produced similar responses, with respect to storage protein accumulation and germination suppression, to those seen with the ABA concentrations (10 and 1 μM, respectively) used in our earlier studies (8). In the absence of sorbitol, embryos at all stages tested germinated within 3 to 4 d (Table I). Germination was inhibited by 8.7% sorbitol up to 41 dpa, but older embryos required increasingly drying conditions to suppress germination, similar to the situation occurring normally in seeds. Neither concentration of sorbitol could prevent germination of mature dry seed embryos. The water potential of air-dry rapeseed is approximately −4000 bars (19), so presumably the water potential gradient favored imbibition of these seeds.

Cruciferin Synthesis in Cultured Embryos. High osmoticum (0.35 M) stimulates synthesis of the legumin-like storage protein, cruciferin, in young embryos (4). Therefore, accumulation of cruciferin and its mRNA were used as markers of sensitivity to osmoticum. Cruciferin levels were measured by rocket immu-

Table I. *Germination of Embryos Cultured on Various Concentrations of Sorbitol*

After 3 d of culture, embryos were scored for the appearance of root hairs.

Stage	After 3 d on		
	No sorbitol	8.7% sorbitol	12.5% sorbitol
dpa		%	
27	97	0	0
33	72	0	0
37	80	0	0
41	100	0	0
46	100	81	0
Dry	100	100	75

noelectrophoresis. Levels of cruciferin mRNA were measured by dot blots and standardized relative to total RNA in the sample.

Both cruciferin and its mRNA show a dose response to osmoticum in embryos excised up to 40 dpa (Figs. 1, 2). When cultured on high osmoticum, these predesiccation stage embryos accumulated cruciferin and its mRNA to levels similar to those seen during development *in situ*. At later stages the osmotic treatments appear to be less effective in maintaining cruciferin accumulation to concentrations normally found in the seed, but a dose response is still seen. Cruciferin mRNA levels were also maintained at significantly higher levels in sorbitol-treated desiccation-stage embryos, even when germinating, than in embryos cultured without sorbitol. However, at these later stages (embryos excised after 40 dpa), the highest osmoticum appeared to be supraoptimal for cruciferin mRNA accumulation. These results show that high osmoticum can approximate the effects of the normal seed environment throughout embryo development with respect to its effects on storage protein synthesis.

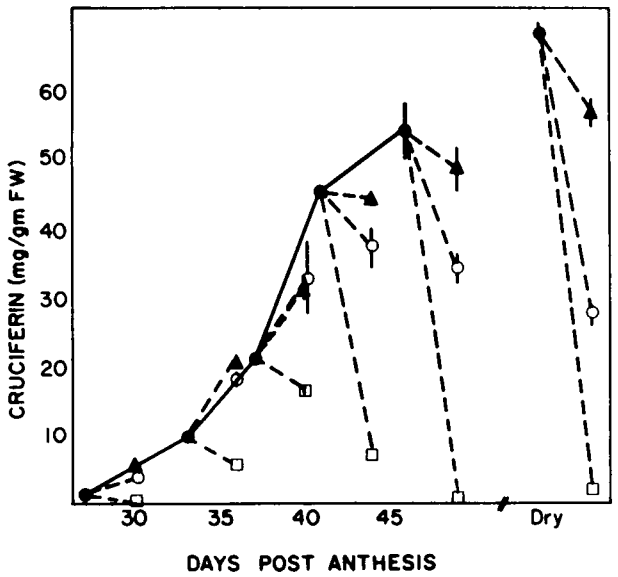

FIG. 1. Cruciferin accumulation in cultured embryos. Embryos of various ages were cultured for 3 d on basal medium with 12.5% sorbitol (▲), 8.7% sorbitol (○), or no sorbitol (□). Cruciferin concentrations were determined by rocket immunoelectrophoresis. The solid lines connect the levels found in embryos 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.

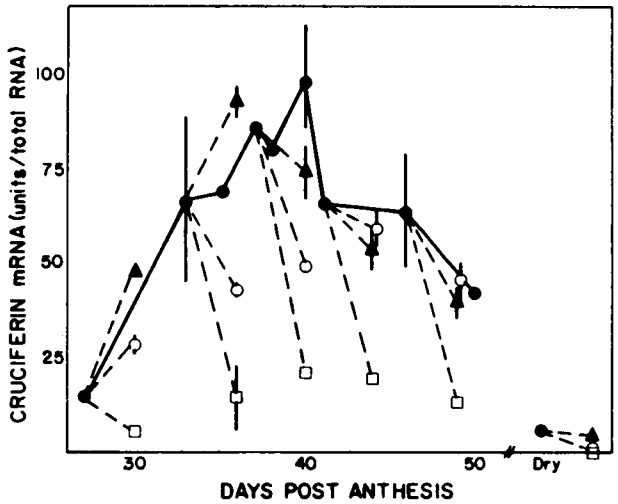

FIG. 2. Cruciferin mRNA in cultured embryos. Embryos of various ages were cultured for 3 d on basal medium with 12.5% sorbitol (▲), 8.7% sorbitol (○), or no sorbitol (□). Cruciferin mRNA levels were determined relative to total cellular RNA by the dot blot procedure. (——), Connect the levels found in embryo development in seeds; (- - -), changes occurring during the culture period. Each point represents the average of duplicate samples, each derived from 10 to 20 embryos. Error bars = SD.

Endogenous ABA in Cultured Embryos. Our previous studies have shown that, in embryos which are rapidly accumulating storage reserves, both suppression of germination and increased storage protein synthesis correlate with high endogenous ABA (8). To test the hypothesis that high osmoticum inhibits germination and maintains embryogeny via increased ABA levels, we measured ABA in embryos cultured under the same osmotic conditions as those used for the sensitivity assays. Surprisingly, the endogenous ABA levels in predesiccation stage (less than 40 dpa) embryos cultured on high osmoticum were much lower than in embryos developing *in situ* (Table II). Although a dose response was seen at all stages, the amount of ABA/embryo decreased several-fold in predesiccation stage embryos under all culture conditions. The fact that high endogenous ABA is not required for suppression of germination and continued storage protein synthesis suggests that ABA probably does not directly regulate these effects.

Kinetics of ABA and Osmotic Effects on Gene Expression. Having determined that high osmoticum could obviate the need for ABA to maintain embryonic development, we wanted to know if the effects of ABA were mediated by inhibition of water uptake. Measurements of water content show that ABA, like culture on high osmoticum, inhibits water uptake (Fig. 3). If this inhibition of water uptake is actually more directly involved than ABA in inhibiting germination and regulating gene expression, its effects should be more rapid. To test this, we compared the kinetics of ABA and osmotic effects on gene expression. Cruciferin mRNA levels decrease to very low levels within 10 h after excision when embryos are cultured on basal medium (5). When subsequently cultured on either ABA or osmoticum, cruciferin mRNA levels increase rapidly. This allows us to compare the timing of reinduction of cruciferin synthesis by ABA or osmoticum. Embryos were cultured for 10 h on basal medium, then transferred to either 10 μM ABA or 10.9% sorbitol for varying intervals before harvesting. These concentrations were chosen such that cruciferin mRNA accumulated to similar levels at the end of culture on either ABA or sorbitol. The timecourse of accumulation of both cruciferin and its mRNA was more rapid following osmotic treatment than on ABA media (Fig. 4). Although this appears to contradict our previous finding that cruciferin synthesis increases more rapidly in response to exogenous ABA than to high osmoticum (4), these experiments dif-

Table II. *Endogenous ABA in Cultured Embryos*

ABA was measured by radioimmunoassay in embryos of various ages cultured for 3 d on basal medium with 12.5%, 8.7%, or no sorbitol. Values represent the average of duplicate assays of at least two samples, each derived from 10 to 20 embryos.

Stage	Upon Excision	Cultured 3 d with		
		No sorbitol	8.7% sorbitol	12.5% sorbitol
dpa		*ng/mg fresh wt*		
27	1.11 ± 0.49	0.17 ± 0.1	0.23 ± 0.06	0.25 ± 0.06
33	1.21 ± 0.09	0.15 ± 0.06	0.22 ± 0.01	0.22 ± 0.04
37	1.28 ± 0.3	0.26 ± 0.1	0.43 ± 0.12	0.72 ± 0.36
38	2.4 ± 0.12	0.29 ± 0.15	0.49 ± 0.31	0.51 ± 0.16
46	0.71 ± 0.33	0.11 ± 0.04	0.38 ± 0.13	0.58 ± 0.17

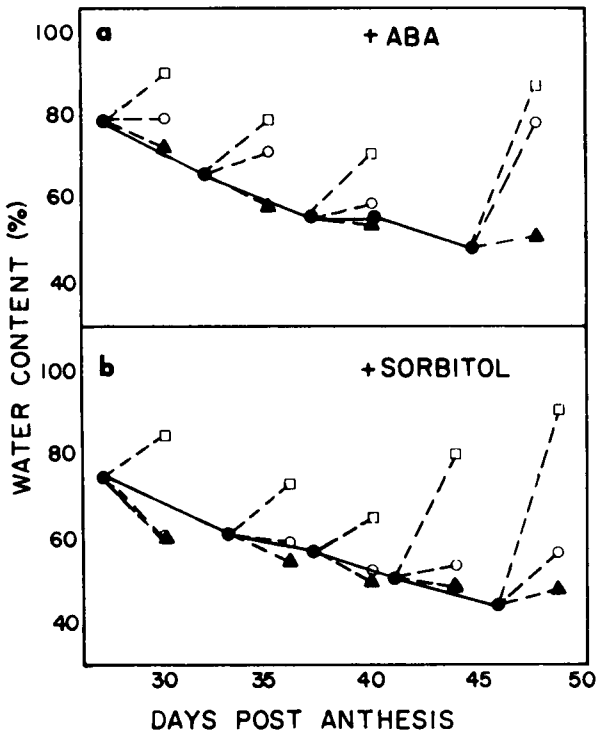

FIG. 3. Water content in cultured embryos. Embryos of various ages were cultured for 3 d with or without ABA (a) or sorbitol (b). In panel a, basal medium was supplemented with 10 μM ABA (▲), 1 μM ABA (○), or no ABA (□). In panel b, the media contained 12.5% sorbitol (▲), 8.7% sorbitol (○), or no sorbitol (□). (——), Connect water contents found during development in seeds; (---), changes occurring during the culture period.

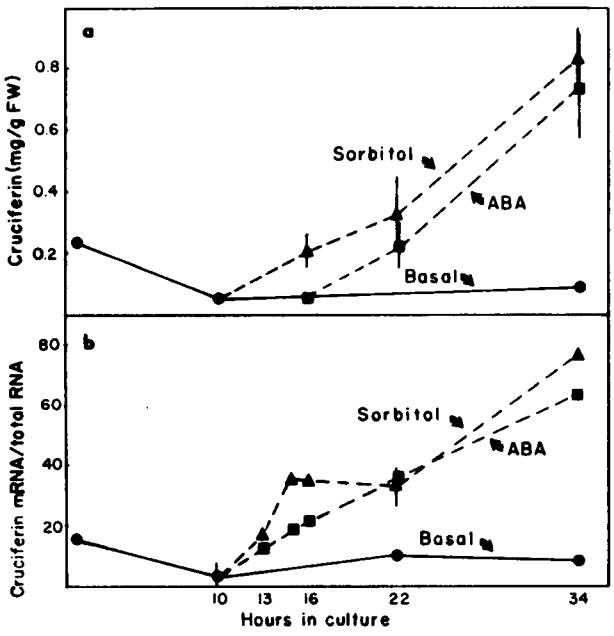

FIG. 4. Kinetics of ABA and osmotic effects on cruciferin synthesis. Embryos were excised at 27 dpa, cultured 10 h on basal medium (●), then some were transferred to either 10 μM ABA (■) or 10.9% sorbitol (▲) for varying periods. Cruciferin concentrations (a) were determined by immunoelectrophoresis. Cruciferin mRNA levels (b) were measured by dot blots. Error bars = SD.

fered in several respects. While the earlier studies measured rates of synthesis after 2 or 5 d in culture, the present studies monitored reinduction of protein and mRNA accumulation over a 24 h period. Measurements of endogenous ABA at 5 and 24 h of reinduction showed that the osmotic effect did not reflect a transient increase in ABA (Fig. 5), supporting the idea that high ABA levels are not necessary for continued cruciferin synthesis.

DISCUSSION

In an effort to identify changes associated with embryo maturation, we have been comparing development *in situ* with development in culture to characterize and mimic conditions in the seed. We have used culture media containing either ABA (8) or high osmoticum because both ABA and drying conditions are a normal part of the seed environment. Our results from the ABA culture experiments suggested that although ABA is important in suppressing germination and maintaining embryonic development during the phase of rapid storage reserve accumulation, low water content is probably more important in regulating the transition to developmental arrest during maturation. Many studies have shown the importance of desiccation in the transition from embryogeny to the ability to germinate and form normal seedlings. Premature drying, followed by rehydration, of *Phaseolus vulgaris* or castor bean embryos results in a shift in developmental potential from embryonic to germinative growth, reflected by changes in patterns of protein synthesis (15). Similarly, immature wheat grains will not produce α-amylase in response to GA_3 unless first sensitized to GA_3 by drying to a critical water content (2). Finally, the timing of the onset of water loss in rapeseed embryos correlates with the transition from the potential for 'continued embryogeny,' exhibited by precociously germinating immature embryos, to the ability to form morphologically normal seedlings (7).

In the present experiments we examined the role of reduced water uptake in regulating embryogeny from early cotyledon stage to maturity. We tested the ability of high osmoticum (0.48 and 0.69 M) to mimic normal development, using suppression

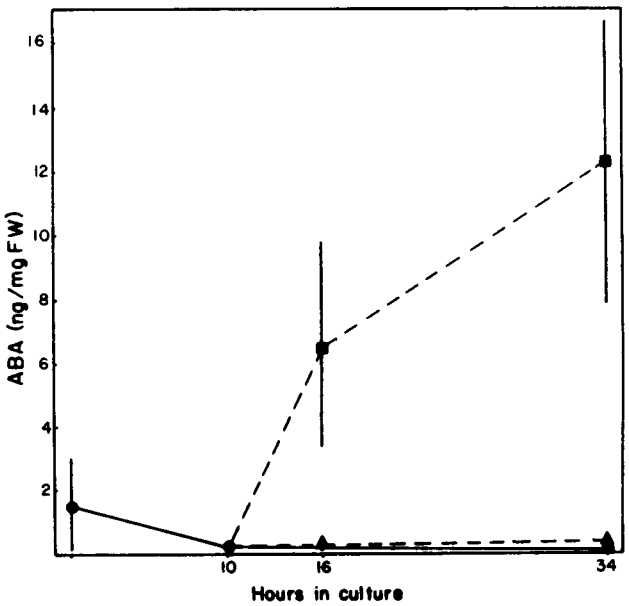

FIG. 5. Endogenous ABA during reinduction of cruciferin synthesis. Embryos were excised at 27 dpa, cultured 10 h on basal medium (●), then some were transferred to either 10 μM ABA (■) or 10.9% sorbitol (▲) for varying periods. ABA was measured by radioimmunoassay. Values represent the average of duplicate assays of at least two samples, each derived from 10 to 20 embryos. Error bars = SD.

of germination and maintenance of storage protein synthesis at levels similar to those in embryos developing *in situ* as criteria. Sorbitol was used as the osmoticum in these experiments and similar results have been obtained with mannitol and sucrose (RR Finkelstein, ML Crouch, unpublished observations); these compounds presumably produce an osmotic, not a nutritional, effect. The concentrations used (8.7 and 12.5%) were chosen empirically for their ability to produce responses in cultured midcotyledon stage embryos similar to those seen with the ABA concentrations used in the earlier studies (1 and 10 μM, respectively). As expected, the sorbitol treatments gave results similar to the ABA treatments with predesiccation stage embryos in that they prevented germination and maintained cruciferin accumulation at levels comparable to embryos developing *in situ*. Cruciferin mRNA accumulation in embryos cultured on osmoticum more closely approximated levels in the seed than did the ABA-treated embryos, which had lower levels of cruciferin mRNA than *in situ* (8). At later stages, the osmotic conditions used were no longer sufficient to prevent the embryos from taking up water and germinating. However, the embryos cultured on sorbitol, even when germinating, maintained significantly higher cruciferin mRNA levels than those in embryos cultured on basal medium.

Although high osmoticum presumably prevents germination by blocking water uptake, it is not clear how it affects gene expression. One possibility is that embryos exposed to high osmoticum, like many other plant tissues, have elevated ABA levels and that ABA mediates the effects on gene expression. Although the source of embryonic ABA in developing rapeseeds is not known, culture studies have shown that wheat embryos (14) and grape pericarp (12) can synthesize ABA in response to osmotic stress, as evidenced by incorporation of label from the ABA precursor, mevalonate. To test whether the effects of osmoticum on rapeseed embryos are mediated by ABA, we measured endogenous ABA in embryos cultured on sorbitol. Although these culture conditions maintained cruciferin mRNA accumulation at higher levels than in the ABA-treated embryos, the endogenous ABA levels were about 10-fold lower in embryos cultured either with or without sorbitol in the medium than in ABA-treated embryos. Bray and Beachy (3) recently reported similar results with cultured soybean cotyledons. Culture on ABA and/or sucrose (up to 5%) resulted in increased β-conglycinin synthesis and ABA levels. These data were interpreted as evidence that ABA regulates cotyledon-specific gene expression. However, the endogenous ABA levels were 30- to 50-fold lower in cultured cotyledons than in those that had not been cultured. The fact that high endogenous ABA is not necessary to inhibit germination and maintain storage protein synthesis suggests that ABA does not directly regulate these processes. Similarly, although the accumulation of proline as osmoticum in response to water stress correlates with the presence of high ABA levels in wilted and ABA-treated barley leaves, salt stress-induced proline accumulation is not preceded by elevated endogenous ABA levels (21). It is also possible that an ABA metabolite which we would not detect by radioimmunoassay, such as phaseic acid, is more directly involved in gene regulation. In barley aleurone layers, phaseic acid is at least as active as ABA in inhibiting GA-enhanced synthesis of α-amylase (11). Furthermore, in barley aleurone ABA stimulates its own conversion to phaseic acid thereby enhancing its apparent effectiveness (22). Another possibility is that the osmotic treatments affect compartmentalization of ABA at either the subcellular or tissue level such that it is present in high concentrations in certain 'target' sites. For example, studies with spinach leaves have shown that osmotic stress results in increased ABA synthesis in mesophyll tissue and that the ABA migrates to the epidermis where it can induce stomatal closure (13). However, during embryo development *in situ* storage proteins are synthesized and accumulate in protein bodies throughout the embryos (20), so it is unlikely that their synthesis is stimulated to very high levels in a small subset of cells, containing a high concentration of ABA, in embryos cultured on sorbitol. Studies with cotton embryos have suggested that 90% of embryonic ABA is localized in the vacuole (10), so the large amounts of ABA normally seen during embryogenesis may be sequestered away from the target site. It is possible that the osmotic treatments deplete the vacuolar pool while leaving ABA at its site of action.

Although high osmotic conditions do not cause an increase in ABA levels in cultured rapeseed embryos, high ABA levels do inhibit water uptake. Inhibition of water uptake by ABA was studied by Schopfer (18) working with mature dry rapeseed. He showed that ABA and osmotic stress appear to act synergistically in suppressing germination suggesting that they may be acting through a common effector, but that ABA inhibition of germination shows a slight lag relative to inhibition by PEG-induced osmotic stress. Similarly, our kinetic studies show that the sorbitol-induced osmotic effect on gene expression is more rapid than the effect of ABA. These results are consistent with the hypothesis that ABA inhibits water uptake, which regulates embryogeny in some unknown manner.

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