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Control of Enzyme Activities in Cotton Cotyledons during Maturation and Germination

I. NITRATE REDUCTASE AND ISOCITRATE LYASE[1]

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ABSTRACT

Actinomycin D at 10 μg/ml strongly inhibited the increase in isocitrate lyase activity during germination of seeds and 40-day-old embryos of cotton (*Gossypium hirsutum* L.) when the germination period was preceded by 3 hours of soaking in the inhibitor solution. No inhibition was observed without the presoaking. Induction of nitrate reductase activity by nitrate was never inhibited by actinomycin D under the same conditions, and was frequently stimulated about 50%. Thus, the method of applying actinomycin D to the seeds and ovules could affect interpretation of its action. Abscisic acid at 5 μg/ml blocked production of isocitrate lyase activity in both pregermination treatments, but did not inhibit induction of nitrate reductase activity. Induction of nitrate reductase activity became insensitive to the two inhibitors during ovule maturation, at about 32 days after anthesis. The results indicate that isocitrate lyase, a germination enzyme, is not synthesized on preformed mRNA. In this respect, the appearance of activity in cotton resembles that in other species of fatty seeds. In contrast, induction of nitrate reductase activity, which is unnecessary for germination, apparently is not regulated at the level of transcription except in young ovules.

In recent years, Dure and co-workers (7-10, 19) and Hammett and Katterman (5) have demonstrated the existence of preformed mRNA in cotton seeds and immature embryos. Ihle and Dure (7-10), using sensitivity to Act D[2] as the criterion for enzyme synthesis on this preformed mRNA, concluded that it coded in part for two "germination" enzymes, isocitrate lyase and carboxypeptidase C. The synthesis of this mRNA was reported to occur during seed maturation, with its subsequent translation prevented by endogenous ABA. Such temporal separation of transcription and translation was considered to be an important regulatory mechanism for enzymes intimately associated with the germination process (2).

Two recent developments have clouded the interpretation of events offered by Ihle and Dure (9). First, Smith *et al.* (17), using somewhat different methods of seed germination, were able to block production of isocitrate lyase activity with Act D. Second, Act D was shown to inhibit mRNA synthesis only 65% in this system (19). Thus, the effectiveness of the inhibitor on synthesis of an individual species of mRNA was open to question.

Cotton seeds are unusual in that nitrate induces considerable transitory nitrate reductase activity during germination (14, 15). Radin (14) showed that the induction was insensitive to Act D. However, his imbibition methods were similar to those of Ihle and Dure (9), rather than those of Smith *et al.* (17). The insensitivity of nitrate reductase induction toward Act D is interesting, because the enzyme is very different from the so-called germination enzymes. Nitrate reductase does not contribute to germination, and in fact, it has been called a "luxury enzyme" during germination (12).

We have reinvestigated the sensitivity of isocitrate lyase synthesis in cotton to Act D, with the intent of reconciling the conflicting reports in the literature (9, 17). In addition, we have characterized the effects of Act D and ABA on nitrate reductase induction in germinating seeds and immature embryos, and compared its regulation by these compounds to the regulation of isocitrate lyase synthesis.

MATERIALS AND METHODS

Chemicals. Act D, dithiothreitol, and ABA were purchased from Sigma Chemical Co.[3] No differences in response to ABA were noted whether the source was *cis, trans,* or mixed isomers.

Sources of Seeds and Ovules. We have found some variation in behavior among seedlots. For these experiments, seeds of cotton (*Gossypium hirsutum* L. cv. Deltapine 16), hand harvested in 1973, were acid delinted before use. Ovules of known ages were obtained from greenhouse-grown plants on which flowers were tagged at anthesis. The embryo fresh weights corresponded closely to those reported by Ihle and Dure (9).

Germination Methods. The major difference between the experiments of Ihle and Dure (9) and those of Smith *et al.* (17) was in the method of imbibition. We thus developed two germination procedures which approximated their conditions. In the first method, comparable to that of Ihle and Dure (9), dry seeds were placed on filter paper in Petri dishes containing either water, ABA, or Act D solutions, and then were incubated in darkness at 30 C. All solutions contained 10 mM or 100 mM KNO_3 and wee filter-sterilized. The dishes and paper were autoclaved before use. Seed coats were removed after 24 hr of

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[2] Abbreviation: Act D: actinomycin D.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

incubation, and embryos were replaced on fresh media. The second method was comparable to that of Smith *et al.* (17). Seeds were soaked in water for 3 hr, the seed coats were removed, and the embryos were transferred to the appropriate inhibitor solution for 3 more hr. At the end of the second soaking period, the imbibed embryos were placed in Petri dishes and incubated as above. Immature embryos were removed from ovules and either put directly on the incubation medium in the Petri dishes, or soaked for 3 hr in the appropriate solution before being placed in the dishes. Incubation times given under "Results" are measured from initial treatments of embryos or dry seeds.

Enzyme Activities. Cotyledons were excised, washed, and ground in a Tenbroeck-type homogenizer in 0.1 M K phosphate buffer, pH 6.9, containing 10 mM $MgCl_2$ and 3 mM dithiothreitol (1 ml buffer/cotyledon pair). The crude homogenate was further disrupted with one pass through a French press at 1265 kg/cm^2 (18,000 p.s.i.), then centrifuged at 27,000*g* for 20 min. Isocitrate lyase activities were assayed in the supernatant (1). An assay method specific for glyoxylate production (16) gave comparable results. Nitrate reductase activity of intact cotyledons was determined by an *in vivo* assay described previously (14).

RESULTS

Our experiments show that Act D can strongly influence seedling growth, and that this influence depends upon pregermination treatment. When either mature seeds or immature embryos were soaked in 10 μg/ml Act D prior to germination, the subsequent radicle growth was inhibited up to 50% or more (Fig. 1). There was no such effect in seedlings which had not been presoaked (Fig. 1). Germination was 95 to 100% for control and Act-D-treated seedlings in both pregermination treatments, and radicle elongation was closely related to the degree of cotyledonary unfolding and enlargement. In contrast, germination, radicle elongation, and cotyledon development were almost totally suppressed by ABA, regardless of pregermination treatment (Fig. 1).

Activity of isocitrate lyase followed the same pattern as did growth. In presoaked seeds, Act D strongly inhibited development of activity, but the drug had little or no effect in seeds that were not pretreated (Table I). The same differential effect of Act D was clearly evident in immature embryos, although the per cent inhibition in presoaked embryos was less than in presoaked seeds (Table I).

In seeds treated with ABA, there was almost no isocitrate lyase activity even after 50 hr of germination (Table I). Pretreatment did not alter this effect. In immature embryos, ABA also inhibited development of activity, but not so severely as in seeds (Table I).

Development of nitrate reductase activity was entirely different from that of isocitrate lyase activity. Peak activities in seeds certainly were not inhibited by Act D with either treatment, and usually showed some increase over the control (Table II). However, presoaking greatly accelerated the development of peak activity. In these seedlings, the greatest activity was present only 6 hr after the start of germination, whereas activity increased until 50 hr in unsoaked seeds (Table II). Presumably, the large displacement of the peak resulted from different rates of water uptake and activation of seed metabolism.

Act-D-treated immature embryos had higher nitrate reductase activity than the controls (Table II). In this experiment, the increase varied from slight (not presoaked) to more than 100% (presoaked). Typically, the stimulation by Act D was quite variable; however, over many experiments, the average activity of Act-D-treated embryos or ovules was about 50% greater than the controls.

As in the case of Act D, ABA affected isocitrate lyase and nitrate reductase activities differently. In unsoaked seeds, ABA effected a small inhibition of induced nitrate reductase activity, ranging up to about 25% (Table II). In presoaked seeds, the peak activity (at 50 hr) was essentially unchanged from the peak

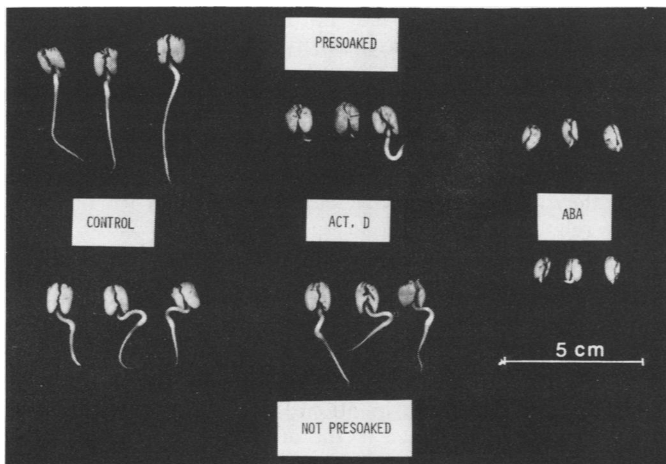

FIG. 1. Mature seeds germinated for 50 hr. Presoaked seeds (upper row) were soaked in water for 3 hr and then transferred to water, 10 μg/ml Act D, or 5 μg/ml ABA for 3 hr more prior to germination.

Table I. *Isocitrate Lyase Activities of Cotyledons from Germinating Seeds and 40-Day-Old Immature Embryos*

Presoaked seeds and embryos were soaked in water for 3 hr and then transferred to water, 10 μg/ml Act D, or 5 μg/ml ABA for 3 hr more prior to germination as described in the text. In all cases, incubation times are measured from initial treatment.

Tissue and Incubation Time	Isocitrate Lyase Activity					
	Presoaked			Not Presoaked		
	Water	Act D	ABA	Water	Act D	ABA
Seeds	nmoles/min/embryo					
6 hr	11	4	---	4	2	---
30 hr	261	63	8	62	58	11
50 hr	348	48	6	380	340	8
Immature Embryos						
30 hr	44	12	---	20	24	---
50 hr	94	63	28	49	51	15

Table II. *Nitrate Reductase Activities of Germinating Seeds and 40-Day-Old Immature Embryos*

Seeds and immature embryos were treated as described in Table I.

Tissue and Incubation Time	Nitrate Reductase Activity					
	Presoaked			Not Presoaked		
	Water	Act D	ABA	Water	Act D	ABA
Seeds	nmoles/hr/embryo					
6 hr	277	281	128	0	0	0
30 hr	246	273	184	84	102*	74
50 hr	17	71	241	252	271*	189
Immature Embryos						
30 hr	75	171	95	103	118	163

* 20 μg/ml Act D

in the control (at 6 hr). ABA stimulated activity in immature embryos regardless of pretreatment (Table II).

Nitrate reductase induction retained its insensitivity to Act D and ABA even under conditions which allowed the drug to inhibit seedling growth and isocitrate lyase development (Fig. 1, Tables I, II). At what stage of ovule development does that insensitivity appear? To answer this question, we studied the effect of the two inhibitors on induction during ovule maturation.

Nitrate could induce nitrate reductase activity in ovules of all ages tested (Fig. 2). The nitrate-induced increase was superimposed upon the activity of the uninduced ovules (water controls), which declined slowly from about 100 nmol/hr·ovule at 21 days after anthesis to about 20 nmol/hr·ovule at 40 days. Induction by nitrate was greatest in the youngest ovules, decreasing rapidly with age until 26 days and slowly thereafter. In the interval from 26 to 40 days, ovules given nitrate had two to four times the activity of the water controls.

Because of the very large changes in induced nitrate reductase activity during development (Fig. 2), the effects of Act D and ABA are reported here as percentages of the nitrate-only activity. When expressed on this basis, the results showed a clear pattern. Both Act D and ABA inhibited induction of activity by nitrate in ovules younger than 32 to 34 days, but stimulated it in ovules older than that (Fig. 3). Between 25 and 30 days after anthesis, control (nitrate-free) activity was about 40% of the nitrate-induced total (Fig. 2). Thus, activity in the presence of Act D or ABA at that stage was approximately the same as basal levels found without nitrate (Fig. 3). In ovules about 40 days old, the increase in induction was again somewhat variable, but was persistent through many trials.

DISCUSSION

In other species of fatty seedlings, development of isocitrate lyase activity is sensitive to Act D during early germination (4, 6). Our data fully support Smith *et al.* (17) in putting cotton into this class of seedlings. Figure 1 and Table I clearly show that the means of applying Act D to seeds and ovules affects interpretation of its action. With a presoak treatment, 10 μg/ml Act D strongly inhibits radicle growth and production of isocitrate lyase activity. In contrast, the effect of ABA does not depend upon pretreatment.

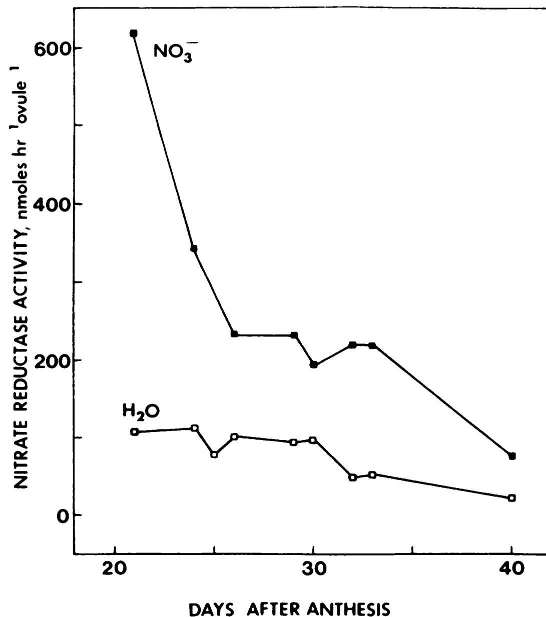

FIG. 2. Nitrate reductase activity of isolated ovules incubated 24 hr on either water or 100 mM KNO_3. Each point is the mean of several trials.

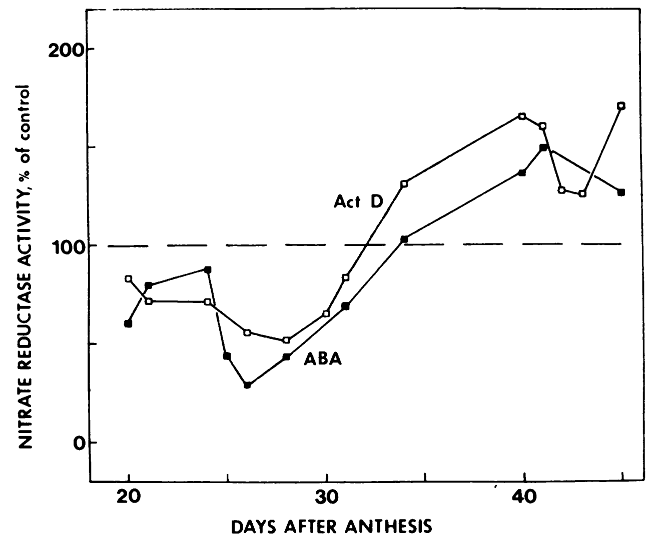

FIG. 3. Nitrate reductase activity of isolated ovules incubated 24 hr on 100 mM KNO_3 with either 10 μg/ml Act D or 5 μg/ml ABA. The values are shown as percentages of the nitrate-only control. Each point is the mean of several trials.

Induction of nitrate reductase activity is not inhibited by Act D under conditions which foster strong inhibition of isocitrate lyase synthesis (Table II). Nitrate reductase activity in seeds reaches maximum levels only 6 hr after initial soaking, whereas isocitrate lyase activity is still negligible (Tables I, II). The absence of an extended lag phase for induction of nitrate reductase activity, and the insensitivity to Act D, are consistent with the possibility of preformed mRNA for the enzyme. Alternatively, substantial nitrate reductase might exist in the dry seed, in either an active or inactive state. This possibility remains untested because the *in vivo* assay technique is not applicable to dry seeds. However, the level of activity in germinating seeds is more than 10-fold greater than that found in ovules as they approach maturity (Fig. 2). Furthermore, activity is zero in unsoaked seeds 6 hr after imbibition, when the assay can be used (Table II), and cycloheximide strongly inhibits induction (14). Taken together, these facts suggest that activity results from *de novo* protein synthesis.

Induction of nitrate reductase activity becomes insensitive to Act D about 32 days after anthesis (Fig. 2). Because recent work (19) indicates that Act D may not inhibit all mRNA synthesis, the reasons for Act D insensitivity can not be deduced with certainty. Regardless of mechanism, the transition to insensitivity occurs at the same time as that reported for carboxypeptidase C (9). The characteristics listed by Ihle and Dure (9) for behavior of carboxypeptidase C thus appear also to describe nitrate reductase, which is not a germination enzyme. On the other hand, they do not apply to isocitrate lyase, which is obviously closely related to germination (Fig. 1, Table I). In cotton, nitrate reductase and carboxypeptidase C may be representative of a class of enzymes whose appearance is Act D insensitive, but this class excludes the unique enzymes of the glyoxylate cycle (18).

Inhibitor studies such as these are frequently difficult to interpret. It could be argued that soaking seeds in Act D or ABA causes its buildup to toxic levels. However, there is no obvious necrosis or flaccidity up to 50 hr, and any toxicity of either compound is not reflected in the activity of nitrate reductase. Since the nitrate reductase assay is an *in vivo* assay which depends upon internal generation of reduced pyridine nucleotides (3, 11, 13), we can conclude that the glycolytic pathway of the cotyledons is still functional. In addition, we have found

other enzyme activities unaffected by Act D or ABA treatment (18).

In conclusion, we have altered the germination procedures of Ihle and Dure (9) in a way which seems to enhance the biological effectiveness of applied Act D. We find that Act D and ABA under these conditions can block increases in isocitrate lyase activity during germination of seeds and immature embryos, but that they do not inhibit induction of nitrate reductase activity. There is no evidence of toxicity. The effects of Act D on nitrate reductase activity are very similar to its reported effects on carboxypeptidase C activity (9). Our results are consistent with the possible existence of preformed message for nitrate reductase, but not for the germination enzyme, isocitrate lyase.

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