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Developmental and Environmental Induction of *Lea* and *LeaA* mRNAs and the Postabscission Program during Embryo Culture

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The major programs of gene expression during late embryogenesis are the maturation or reserve accumulation program and, after ovule abscission, the postabscission program that is composed largely of *Lea* and *LeaA* mRNAs that probably encode desiccation protectants. There are diverse opinions about the developmental regulators of these programs. Several candidates are evaluated here by measuring, in cultured embryos, the accumulation kinetics of cloned mRNAs specifically expressed in the normal maturation, postabscission, or germination programs of cotton. Maturation-stage embryos both terminate the maturation program and induce the postabscission program after excision and culture, just as they do later in the plant after ovule abscission. However, they also induce simultaneously the germination program and are thus different from any normal stage of embryo development or germination. The developmental induction of the postabscission program in culture does not require exogenous abscisic acid, but its expression is enhanced by precocious desiccation or culture on abscisic acid or high osmoticum, probably by an environmentally responsive mechanism that normally operates during germination. Normal desiccation does not control any of these programs because the embryo acquires all of the characteristics of a mature embryo before it desiccates. These and other results suggest regulation of normal embryogenesis by a maternal maturation factor, a postabscission factor, and the postabscission program.

INTRODUCTION

There remain several broad questions concerning the regulation of plant embryogenesis. Still unclear is the significance of a simple observation. Most immature embryos are capable of germination (axis elongation and cotyledon unfolding) in culture after cell division is completed. Rather than germinate in the ovule, the immature embryo continues normal development on the plant, first accumulating reserve proteins and lipids during the maturation stage and then putative desiccation protectants during the postabscission stage that follows ovule abscission. The embryo finally desiccates to become a quiescent mature embryo. Removing the developing embryo from the maternal environment and placing it in culture has been regarded widely, if not critically, as an appropriate experimental system to study putative endogenous regulators of embryogenesis and germination. The principal reason is that precocious germination of immature embryos in culture can usually be inhibited by culture with exogenous abscisic acid (ABA), a plant growth regulator that has been implicated independently as an endogenous regulator of embryogenesis by the phenotypes of certain mutants (reviewed by Raghavan, 1986; Crouch, 1987; Galau et al., 1991).

By following molecular markers of putative embryonic and germination programs of gene expression in cultured embryos, previous studies have implicated variously ABA (Dure et al., 1981; Quatrano et al., 1983; Finkelstein et al., 1985; Bray and Beachy, 1985; Eisenberg and Mascarenhas, 1985; Sánchez-Martínez et al., 1986; Williams and Quatrano, 1988), minimal water contents (Finkelstein and Crouch, 1986; Galau et al., 1987; Rosenberg and Rinne, 1988; Bewley et al., 1989), ovule abscission (Galau et al., 1987; Hughes and Galau, 1989), and desiccation (Kermode and Bewley, 1986) as being regulators of embryogenesis. Unfortunately, the roles of these factors have not been resolved, primarily because only a small number of markers have been used in limited experiments of uncertain relevance (Crouch, 1987; Galau et al., 1991).

Particularly troubling is the reported dependence on exogenous ABA of the precocious induction of most *Lea* and *LeaA* mRNAs in cultured cotton embryos (Dure et al., 1981; Galau et al., 1986) and at least some individual *Lea*-like mRNAs in other cultured embryos (Williamson et al.,

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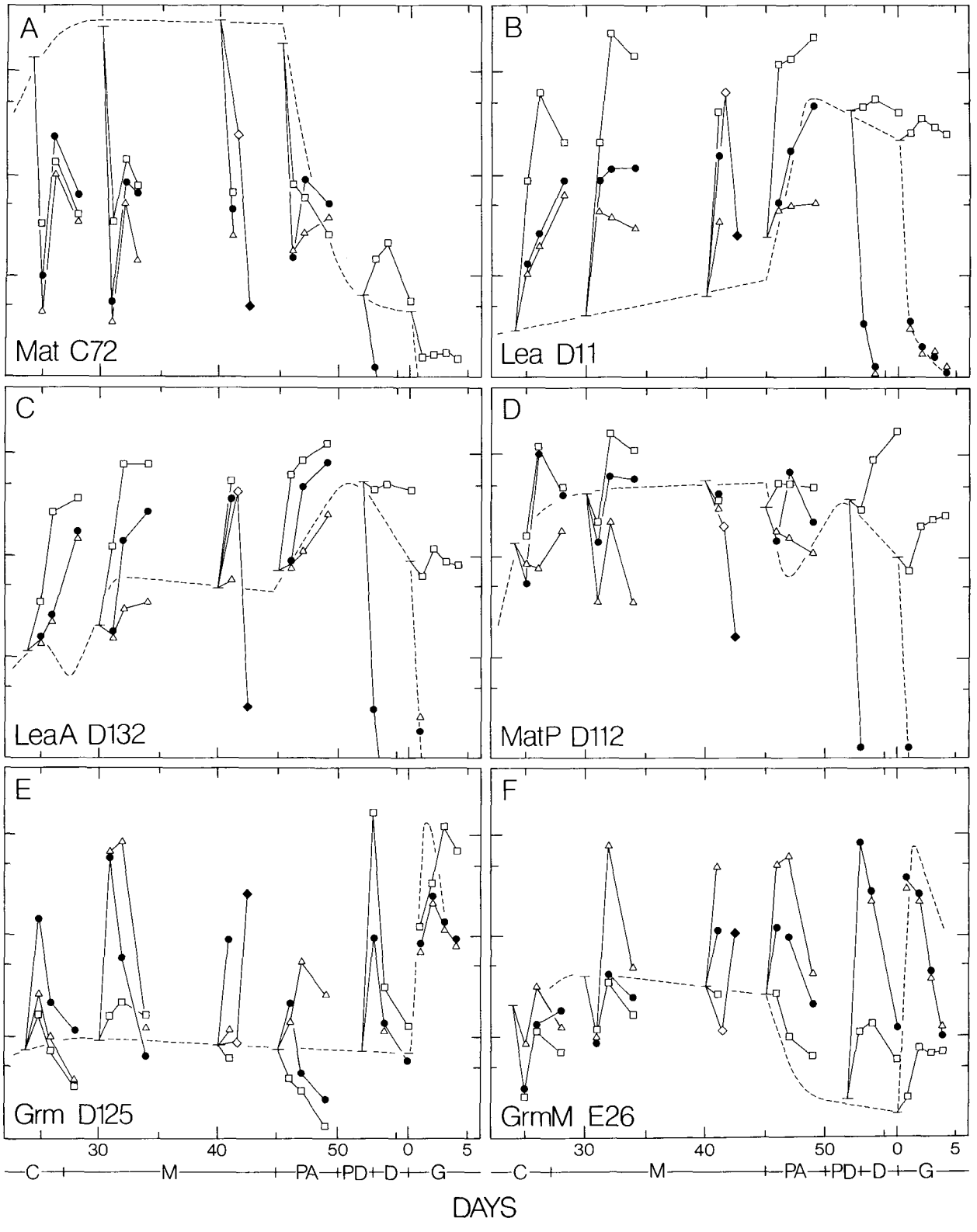

Figure 1. Concentration of Representative mRNAs in Cotyledons of Cultured Embryos.

1985; Gómez et al., 1988; Mundy and Chua, 1988; Harada et al., 1989; Vilardell et al., 1990). In cotton these mRNAs compose the bulk of the postabscission program (Hughes and Galau, 1989) and have been suggested to be desiccation protectants (Galau et al., 1987). In the plant, their major expression is induced by ovule abscission at a time much later than during the highest endogenous ABA concentration and declining water potential that occur during early maturation (Galau et al., 1987; Hughes and Galau, 1989). The same sequence of endogenous events probably occurs in other dicots, and it clearly contradicts the results seen in culture with exogenous ABA (reviewed by Galau et al., 1991; see below). At least in monocots, some *Lea*-like mRNAs have been reported recently to be induced in vegetative organs by ABA and water stress (reviewed by Skriver and Mundy, 1990), again suggesting that their induction in cultured embryos by the same agents need not at all relate to regulation of their developmental expression during embryogenesis in the plant.

To provide some negative and positive controls to evaluate the expression of *Lea* and *LeaA* mRNAs in culture, we recently described many other classes of cotyledon mRNAs that are expressed coordinately during normal development and germination of cotton embryos. Their expression is modular with abundance variously associated with the sequential cotyledon, maturation, postabscission, and germination stages or with transiently high ABA during the first half of the maturation stage (Hughes and Galau, 1989). Although most mRNA classes are expressed as part of several of these programs of gene expression, there are three to 12 mRNAs specific for each of the maturation, postabscission, and germination programs. These mRNAs are used here to address the regulation of mRNA expression and embryo development by defining the competence of immature embryos to express these programs in culture.

The results show clearly that the maturation-stage embryo terminates its maturation program upon excision and culture and simultaneously induces both its postabscission and germination programs. Induction of the postabscission program does not require exogenous ABA, but rather its excision-induced expression is enhanced by exogenous ABA and precocious desiccation, in what is almost undoubtedly an environmental response normally operating during normal germination. Furthermore, these experiments fail to identify any role for desiccation in the control of gene expression or the quality of germination: by all criteria applied, predesiccation-stage embryos are mature before they desiccate.

RESULTS

Precocious Germination and mRNA Expression during Embryo Culture

Forty-seven cDNA clones are available from cotton cotyledons, many of which are useful in defining the developmental programs that are executed in embryos in the plant or in culture (Hughes and Galau, 1989). The nomenclature of the mRNA classes reflects the programs in which each of their mRNAs is modulated during normal development. Thus, *Mat* (maturation), *Lea* (late embryogenesis-abundant, postabscission), and *Grm* (germination) classes have a suffix *C* (cotyledon stage), *A* (endogenous ABA-associated), *M* (maturation), or *P* (postabscission) if they are also expressed in these programs (Hughes and Galau, 1989). Definitions of these developmental stages are those of Galau et al. (1991) and their durations are indicated in Figure 1.

Mature embryos at 56 standard days postanthesis (DPA) germinate rapidly with axis elongation starting by 12 hr and cotyledon unfolding by 24 hr. On a basal medium formulated empirically to best support precocious germination, axis elongation usually starts by 24 hr in 27 DPA early maturation-stage embryos and by 12 hr in 48 DPA postabscission-stage embryos. The ability to green rapidly and uniformly in light also increases with embryo age.

Figure 1. (continued).

The concentration of each mRNA in total RNA during normal development and germination on water (dashed curve) is presented on an arbitrary logarithmic scale as a function of standard days postanthesis and day of germination in water (Hughes and Galau, 1989).
(A) *Mat* C72.
(B) *Lea* D11.
(C) *LeaA* D132.
(D) *MatP* D112.
(E) *Grm* D125.
(F) *GrmM* E26.
Also shown is the mRNA concentration in embryos that were cultured on basal medium (●), 1 μM ABA (□), or 1 μM GA (△), or subjected to precocious desiccation over 1.5 days (◇) and subsequent rehydration and culture for 1 day on basal medium (◆). Abbreviations of developmental stages are: C, cotyledon stage; M, maturation; PA, postabscission; PD, predesiccation; D, desiccation; G, germination (Galau et al., 1987, 1991; Hughes and Galau, 1989).

However, it is not until about 50 DPA to 51 DPA that germination of nondesiccated immature embryos is virtually identical in all respects to that of mature embryos (data not shown).

Although there are at least some similarities in the germination behavior of mature and immature embryos, preliminary experiments using many cloned mRNA markers demonstrated consistently that cultured immature embryos were profoundly different in the expression of their developmental programs from either germinating mature embryos or any stage of immature embryo developing in the plant. Consequently, the accumulation kinetics of all 47 cloned mRNAs was then quantified in cotyledons of embryos at 24, 29, 30, 40, 45, 50, 51, 52, 53, and 56 DPA that were cultured under most of the standard conditions described below, thus surveying embryos from the late cotyledon stage through the mature embryo stage. Figure 1 details the kinetics of representative mRNAs in several of these experiments (solid lines) coplotted with their accumulation during normal embryogenesis and germination on water (dashed curves), as described earlier by Hughes and Galau (1989). Similar behavior in culture was noted for all members of each mRNA class in embryos from 24 DPA until at least 48 DPA. That is, coordinate expression of particular mRNAs during normal development predicted accurately the coordinate expression of the same mRNAs under all culture conditions. To document this, gel blots in Figure 2 show the abundance of all 47 mRNAs in several of the RNA preparations that illustrate, as best as possible, the kinetics of their expression in these experiments.

In presenting the results, first we define the competence of excised embryos on basal medium to execute the gene expression programs that were defined during development (Hughes and Galau, 1989). We then present the results of deliberate attempts to modify expression of these programs with exogenous growth regulators and high osmoticum. Because several changes occur in the response of cultured embryos of a stage just before normal desiccation, first we discuss results with the maturation-stage and postabscission-stage embryo and then those with the predesiccation-stage and mature-stage embryo. Finally, the effect of precocious desiccation on gene expression is examined.

Cultured Maturation-Stage Embryos Terminate the Maturation Program and Induce Both the Postabscission and Germination Programs

The five maturation program-specific mRNAs include those from several vicilin storage protein genes all recognized by *Mat* C72, two legumin storage protein genes each specifically recognized by *Mat* C94 and C134, respectively (Borroto and Dure, 1987; Galau et al., 1988), and a methionine-rich storage proteinlike gene, *Mat* C164 (D.W. Hughes and G.A. Galau, unpublished data). Their levels in maturation-stage embryos are about 1000-fold (C72), 4000-fold (C94, C134, and CD67), and 25,000-fold (C164) higher than in mature embryos (Hughes and Galau, 1989). None of the five *Mat* mRNAs is maintained in cultured 24 DPA to 45 DPA embryos but rather all immediately and rapidly decline in concentration (Figure 1A, closed circles; Figure 2, lanes 2). The initial rates of these excision/culture-associated declines in *Mat* mRNAs are very similar to those observed in normal embryos in the plant after ovule abscission occurs at 45 DPA (Figure 1A, dashed curve). Within 4 days of culture, the levels of all *Mat* mRNAs fall 300-fold to 1500-fold to levels less than 20-fold higher than exist in mature embryos, although there is a transient twofold increase in C134 and C164 mRNAs and a 10-fold to 20-fold increase in C72 and C94 mRNAs during the decline (Figure 1A; Figure 2, lanes 3; data not shown).

The postabscission program clearly is induced in the same cultured embryos. The 12 postabscission-specific *Lea* mRNAs are initially very low in 24 DPA to 45 DPA embryos before culture (Hughes and Galau, 1989; Figure 1B; Figure 2, lanes 1). They immediately increase in cultured embryos (Figure 1B; Figure 2, lanes 2 and 3) at rates and extents both very similar to those seen with *Lea* mRNAs in the plant after ovule abscission occurs at 45 DPA (Hughes and Galau, 1989; Figure 1B; Figure 2, compare lanes 3 and 10). The behavior of the six *LeaA* mRNAs is very similar to that of the 12 *Lea* mRNAs in the plant after 45 DPA (Hughes and Galau, 1989; Figures 1B and 1C), even though the earlier 25 DPA and 32 DPA abundance components of *LeaA* mRNAs in the plant are correlated with the transient maxima in endogenous free ABA (Galau et al., 1987). Significantly, the behaviors of *Lea* and *LeaA* mRNAs are indistinguishable in cultured embryos of any age (Figures 1B and 1C; Figure 2). This similarity is predicted if the expression of both *Lea* and *LeaA* mRNAs is postabscission specific in culture as well as in the plant after 45 DPA. Finally, the increase of *Lea* and *LeaA* mRNAs that occurs in the plant in 46 DPA to 48 DPA postabscission-stage embryos is maintained when these embryos are placed in culture on basal medium (data not shown).

MatP mRNAs decline in the plant like *Mat* mRNAs upon ovule abscission at 45 DPA, but then accumulate like *Lea* and *LeaA* mRNAs during the postabscission stage (Hughes and Galau, 1989; compare Figure 1D with Figures 1A, 1B, and 1C). This suggests that *MatP* mRNAs are regulated independently by both maturation-specific and postabscission-specific events (Hughes and Galau, 1989). Consistent with this interpretation of their behavior in the plant, at least three of the four *MatP* mRNAs in 24 DPA to 45 DPA embryos decline immediately in culture like *Mat* mRNAs (compare Figure 1D with Figure 1A; Figure 2, lanes 2; data not shown). All *MatP* mRNAs then increase in culture like *Lea* and *LeaA* mRNAs to levels near those that are reached during normal postabscission (compare Figure 1D with Figures 1B and 1C; Figure 2, compare

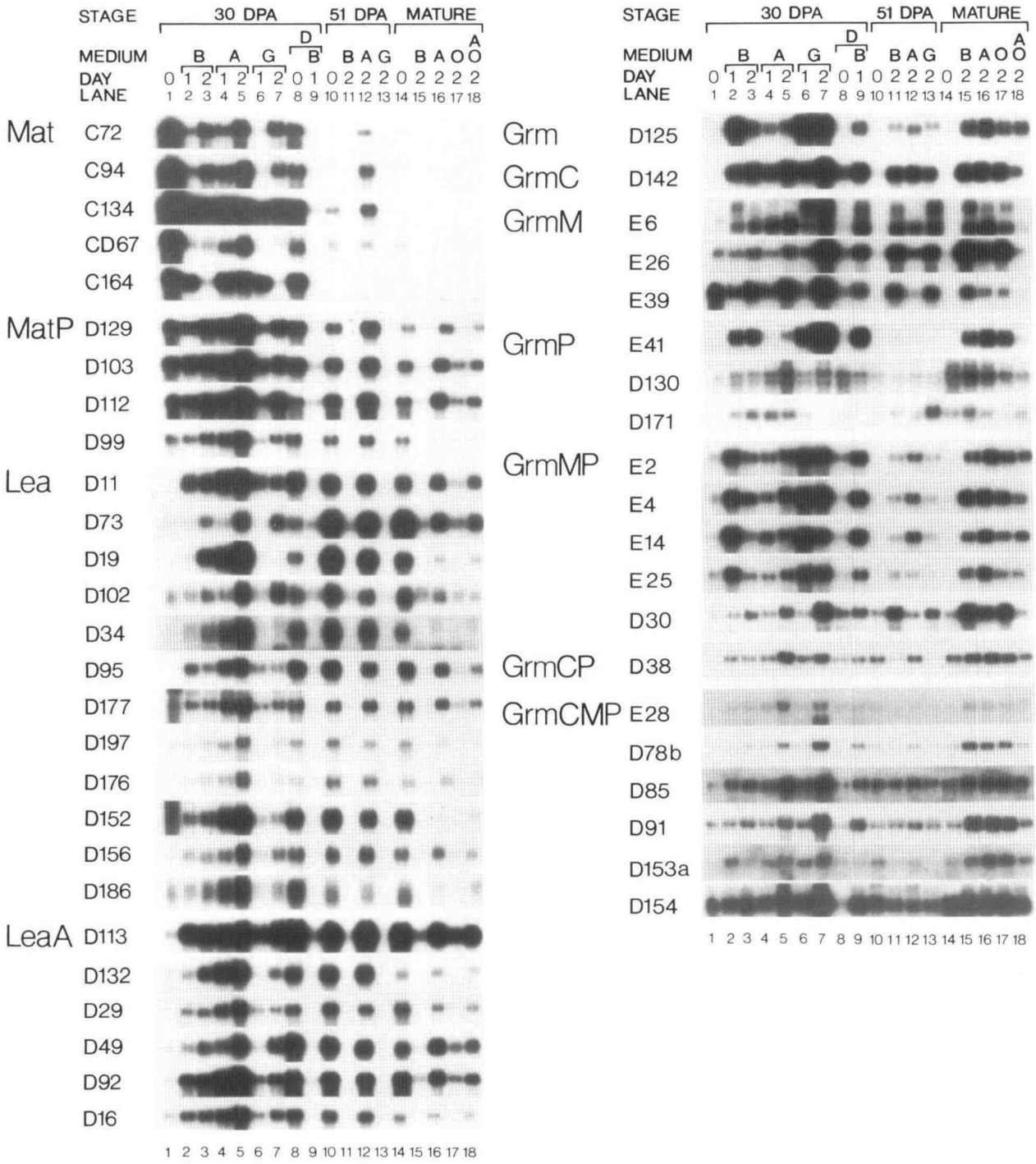

Figure 2. Gel Blot Hybridization of Representative Cotyledon RNAs with All mRNA Probes.

Shown are the results of hybridization to total RNA from embryos of three stages of development: maturation stage (30 DPA), predesiccation (51 DPA), and mature, before culture (0 days); after direct culture for 1 day or 2 days on basal medium (B), 1 μM ABA (A), 1 μM GA (G), OSM (O) containing 6% mannitol, or ABA + OSM (AO) containing 1 μM ABA and 6% mannitol; after precocious desiccation (D) and subsequent rehydration and 1 day of culture on basal medium (B). The 30 DPA embryos here are the same as in Figure 1.

lanes 3 and 10). Late cotyledon-stage and maturation-stage embryos are thus competent to express the postabscission program but require ovule abscission in the plant or the embryo excision/culture actually to do so.

The germination program is also induced in cultured 24 DPA to 53 DPA embryos. Seven classes of germination-abundant mRNAs all have transiently high levels in cotyledons during the first days of normal germination and seedling growth (Hughes and Galau, 1989). The high expression of *Grm* D125, *GrmC* D142, and *GrmP* E41 is essentially germination specific (Hughes and Galau, 1989; Figure 1E; Figure 2). In 24 DPA to 53 DPA embryos cultured on basal medium, these three mRNAs rapidly and transiently increase 20-fold to 100-fold to at least 20% of the level achieved during normal germination on water or basal medium (Figure 1E; Figure 2, compare lanes 1 to 3 with lanes 15). Other germination mRNAs are more highly expressed in the plant before germination, this being attributed to independent expression in one or more of the cotyledon-stage, maturation, or postabscission programs. Specifically, the three *GrmM* mRNAs have major expression during maturation, and the five *GrmMP* mRNAs have minor to modest expression during both maturation and postabscission (Hughes and Galau, 1989; Figure 1F; Figure 2). Several of these mRNAs exhibit the predicted excision/culture-associated decline of maturation-stage mRNAs in cultured 24 DPA to 45 DPA embryos, and they all, along with *GrmP* D130 and D171 mRNAs, exhibit rapid transient increases in cultured 24 DPA to 53 DPA embryos characteristic of germination-specific mRNAs rather than postabscission-specific mRNAs (Figure 1F; Figure 2; data not shown). Although the expression of all six *GrmCMP* mRNAs is coordinate in cultured embryos (Figure 2; data not shown), their expression and that of the remaining germination-abundant mRNA, *GrmCP* D38, cannot be attributed reasonably to either the excision/culture-induced postabscission or germination programs because these particular mRNAs are all expressed to near-equal extents in both of these programs in the plant (Hughes and Galau, 1989). In conclusion, however, the consistent expression of at least 11 germination-abundant mRNAs shows clearly that embryos as early as the late cotyledon stage at 24 DPA express the germination program when placed in culture.

These results may be summarized simply. The coordinate behavior of mRNAs in cultured maturation-stage embryos is entirely consistent with their expression in the inferred developmental programs in the plant and during germination (Hughes and Galau, 1989). Excised maturation-stage embryos in culture turn off the maturation program and initiate and maintain the postabscission program as predicted if excision and culture are experimentally equivalent to ovule abscission in the plant. However, they also execute the germination program in culture. Simultaneous expression of both germination and postabscission programs make cultured cotyledon-stage, maturation-stage, and postabscission-stage embryos significantly different from embryos of any stage of normal development.

ABA and Gibberellic Acid Affect Expression of the Postabscission and Germination Programs

Exogenous ABA at 1 μM (4 μM mixed isomers) completely inhibits germination of 24 DPA to 48 DPA embryos (radicle elongation rate is 50% of control at about 0.1 μM ABA), but only slightly inhibits 50 DPA to 53 DPA and mature embryos (radicle elongation rate is 50% of control at about 1 μM ABA). A stage-dependent inhibition of germination, similar to that seen with 1 μM ABA, also occurs on high osmolarity media (OSM) containing 6% to 12% mannitol. The combination of ABA + OSM, however, completely and reversibly inhibits germination of mature embryos. Gibberellic acid-3 (GA) at 1 μM only slightly increases the rate of germination of embryos of all stages, and in ABA + GA medium it does not reverse the ABA inhibition of germination (data not shown). As described below, 1 μM ABA and 1 μM GA have essentially opposite effects on the expression of most mRNAs in cultured 24 DPA to 48 DPA embryos.

The postabscission-specific *Lea* mRNAs accumulate in 24 DPA to 48 DPA embryos upon excision and culture significantly more rapidly and to higher levels on 1 μM ABA medium (Figure 1B, open squares) than on basal medium (Figure 1B, closed circles; Figure 2, compare lanes 1 and 2 with lanes 4 and 5). These levels often exceed the maximum level achieved in the plant at about 50 DPA. GA at 1 μM reduces the rate and extent of the induction of *Lea* mRNAs (Figure 1B, open triangles) that is otherwise seen on basal medium (Figure 1B, closed circles; Figure 2, compare lanes 2 and 3 with lanes 6 and 7). The behaviors of *Lea* and *LeaA* mRNAs are again virtually identical in all of these culture conditions (Figures 1B and 1C; Figure 2, lanes 1 to 7). *Mat* mRNA expression is apparently affected by these hormones, but to a much lesser degree. Both the rate and extent of the excision-associated decline of *Mat* mRNAs (and *MatP* and *GrmM* mRNAs) are reduced slightly by ABA and enhanced slightly by GA (Figures 1A, 1D, and 1F; Figure 2, lanes 1 to 7). The magnitude of the hormone sensitivities of *MatP* expression after several days in culture is clearly that of *Lea* mRNAs rather than *Mat* mRNAs (compare Figure 1D with Figures 1A and 1B; Figure 2, lanes 1 to 7), consistent with its later expression in culture as being a part of the induced postabscission program.

Conversely, the germination program expressed in cultured 24 DPA to 48 DPA embryos is enhanced by GA and suppressed by ABA. The germination-specific *Grm* D125, *GrmC* D142, and *GrmP* E41 mRNAs (and the three *GrmM*

and five *GrmMP* mRNAs) are all increased significantly by GA and reduced by ABA, usually without greatly affecting their transient kinetics (Figures 1E and 1F; Figure 2, lanes 1 to 7). For those mRNAs that have both significant postabscission and germination components during normal development (*GrmCP*, *GrmCMP*, *GrmP* D130 and D171), both ABA and GA when used alone usually increase expression somewhat above that seen on basal medium (Figures 1E and 1F; Figure 2, lanes 1 to 7), suggesting that these mRNAs are induced as part of both programs.

The enhancement of the postabscission program in cultured maturation-stage embryos by exogenous ABA shows a concentration dependence. Table 1 shows that for most *Lea* and *LeaA* mRNAs examined, increasing exogenous ABA from 1 μM to 5 μM leads to about a 1.5-fold increase in the rate of accumulation of *Lea* and *LeaA* mRNAs. Other experiments have shown that culture on 0.025 μM ABA enhances the rate and extent of *Lea* and *LeaA* mRNAs approximately 1.5-fold over those in culture on basal medium, to about one-fourth those on 1 μM ABA (data not shown). These experiments suggest that half-maximal enhancement by ABA probably occurs at about 1 μM exogenous ABA and that each mRNA has a somewhat different ABA dose-mRNA response relationship and a maximum response relative to the normal level reached in the plant. In more limited experiments (Table 1; data not shown), culture on OSM containing 6% to 12% mannitol produces the same effects as culture on 1 μM ABA. A dose-response relationship also is evident with exogenous GA. Increasing GA from 1 μM to 10 μM further reduces, but by less than a factor of two, the excision/culture-induced postabscission program (data not shown). In summary, no physiologically relevant concentration of exogenous GA abolishes the induction of the postabscission program, and no reasonable concentration of ABA or OSM maintains the maturation program or abolishes the induction of the germination program.

Induction of the Postabscission Program Cannot Be Attributed to Synthesis of ABA

Because the above experiments show clearly that exogenous ABA enhances induction of the postabscission program in maturation-stage embryos, it could be argued that the induction of the program in embryos cultured on basal medium is due to accumulation of endogenous ABA. This possibility is discounted by two considerations. First, Table 1 shows that endogenous ABA does not accumulate significantly in these embryos on basal medium. In fact, it declines rapidly during culture, as has been reported for embryos of soybean (Ackerson, 1984; Bray and Beachy, 1985) and rapeseed (Finkelstein et al., 1985). This decline is reversed significantly in sibling embryos that are cultured on concentrations of ABA sufficient to enhance induction of the postabscission program over that induced on basal medium alone (Table 1). Second, Table 2 shows that the induction of representative *Lea* and *LeaA* mRNAs is unaffected in embryos that are cultured on basal medium supplemented with fluridone, a potent inhibitor of ABA accumulation in other species at the concentrations used here (Moore and Smith, 1984; Bray and Beachy, 1985; Raikhel et al., 1987). The presence of fluridone does not affect the competence of embryos to respond to exogenous ABA (Table 2).

Embryos Are Essentially Mature before They Desiccate

Termination of the Postabscission Program Occurs before Desiccation

Lea, *LeaA*, and *MatP* mRNAs continue to increase in cultured 46 DPA to 48 DPA embryos at rates and to

Table 1. ABA and Postabscission mRNA Levels in Cultured Maturation-Stage Embryos

Embryo Treatment	Endogenous ABA		mRNA Abundance Relative to Maximum in Vivo Level[a]								
			Lea				*LeaA*				
	nmol/g, dry wt	nmol/g of water	D11	D19	D95	D152	D29	D49	D92	D113	D132
28 DPA, excised	4.8	2.5	0.007	0.0006	0.009	0.035	0.006	0.023	0.012	0.007	0.01
2-Day culture											
Basal medium	2.2	0.49	0.4	0.09	0.1	0.17	0.2	0.4	0.55	0.4	0.6
1 μM ABA	8.6	2.3	2.1	1.2	0.17	0.8	0.5	0.45	2.3	0.6	1.2
5 μM ABA	21	6.3	4.0	1.5	0.31	1.0	1.0	0.4	4.1	0.8	2.6
6% Mannitol	2.6	0.61	1.2	3.0	0.14	1.2	1.2	0.6	2.5	0.3	0.6

[a] Maximum in vivo level is defined as 1.0.

Table 2. Fluridone Does Not Inhibit Induction of Postabscission mRNAs in Cultured Maturation-Stage Embryos

	mRNA Abundance Relative to Maximum in Vivo Level[a]						
	Lea			*LeaA*			
Embryo Treatment	D11	D19	D152	D29	D92	D113	D132
33 DPA, excised	0.009	0.008	0.04	0.03	0.05	0.08	0.12
2-Day culture							
Basal medium	0.8	0.08	0.2	0.18	0.7	2.4	0.7
0.3 μM fluridone	0.5	0.06	0.15	0.15	0.3	2.2	0.6
3 μM fluridone	0.9	0.07	0.25	0.2	0.8	2.5	0.7
30 μM fluridone	0.8	0.07	0.18	0.19	0.7	1.8	0.65
1 μM ABA	1.6	0.3	0.4	0.35	2.3	2.1	1.5
1 μM ABA, 0.3 μM fluridone	1.7	0.25	0.4	0.4	2.1	1.9	1.7
1 μM ABA, 3 μM fluridone	1.5	0.4	0.45	0.45	2.5	1.9	2.0
1 μM ABA, 30 μM fluridone	2.2	0.3	0.5	0.45	2.2	2.8	1.6

[a] Maximum in vivo level is defined as 1.0.

extents similar to those seen in the plant (data not shown). When excised just before the desiccation stage, however, 50 DPA to 53 DPA embryos cannot maintain the existing level of postabscission mRNAs during culture. These mRNAs disappear at the same rate as seen in cultured mature embryos (Figures 1B, 1C, and 1D; Figure 2, compare lanes 10 and 11 with lanes 14 and 15), even though there is no evidence of any change in embryo water potential until desiccation starts at 53 DPA (Galau et al., 1987; other data not shown). Similar declines in mRNA abundance are often seen with *GrmCP* and *GrmCMP* mRNAs (Figure 2, lanes 10 and 11), but they are more variable because of their simultaneous induction under the germination program whose induction, as assayed by the three germination-specific mRNAs *Grm* D125, *GrmC* D142, and *GrmP* E41 (Figure 1E; Figure 2, lanes 10 and 11), is unaffected in embryos of this age. It is significant that virtually all postabscission mRNAs also decline in the plant in embryos of the same age of 50 DPA to 53 DPA (Hughes and Galau, 1989; Figures 1B and 1C), indicating as well that the postabscission program must be terminated in the plant at 50 DPA, although its mRNAs apparently are more stable in the plant than in culture. Using the criterion of the decline of postabscission-program mRNAs both in the plant and in culture, we now term the period 50 DPA to 53 DPA the "predesiccation stage" to differentiate it from the completed postabscission stage and the subsequent desiccation stage.

Exogenous ABA Can Restart the Postabscission Program in Cultured Predesiccation-Stage and Mature-Stage Embryos

The maturation program is terminated in the plant at 45 DPA, and the results above show that the postabscission program is terminated in the plant at 50 DPA. As noted above, the remaining levels of *Mat*, *MatP*, *Lea*, and *LeaA* mRNAs of these programs decline rapidly during culture in both 50 DPA to 53 DPA predesiccation-stage and mature embryos on basal medium. In both stages of embryos, however, culture on ABA clearly maintains all these mRNAs at significant levels, although often after an initial decline. Many actually increase 1.5-fold to fivefold in concentration relative to their levels before culture or after 1 day of culture on ABA. These increases are evident especially with *Mat* C72 and C94 mRNAs, all *MatP* mRNAs, and most *Lea* and *LeaA* mRNAs in predesiccation-stage embryos and with all *MatP* and nearly all *Lea* and *LeaA* mRNAs in mature embryos (Figures 1A, 1B, 1C, and 1D; Figure 2, compare lanes 10 and 12 and lanes 14 and 16).

These increases in mRNA levels upon culture with ABA must be due primarily to an increase in transcription or the efficiency of transcript processing, although increased stability could contribute to a minor extent. Increases in both transcription and mRNA stability have been implicated to occur in ABA-treated, mature wheat embryos (Williamson and Quatrano, 1988). It is possible, of course, that ABA enhances induction of the postabscission program in cultured younger embryos by similar mechanisms.

Novel Hormone Sensitivities of Mature Embryos Are Acquired before Desiccation

In addition to having terminated the postabscission program in the plant before desiccation, 50 DPA to 53 DPA predesiccation-stage embryos also express in culture two novel hormone responses that are seen in cultured mature embryos but not seen in younger maturation-stage or postabscission-stage embryos. The most striking is the

enhancement of expression by ABA of *Grm* D125 and at least four of the five *GrmMP* mRNAs in both cultured predesiccation-stage and mature embryos (Figure 1E; Figure 2, lanes 11 and 12, lanes 15 and 16), quite opposite to their inhibition by ABA in younger embryos (Figure 1E; Figure 2, lanes 3 and 5). For the *GrmMP* mRNAs, this increase is far greater than can be attributed to the presumed ABA-induced maintenance or enhancement of the postabscission expression of these mRNAs, and for both *Grm* and *GrmMP* mRNAs the transient kinetics of the ABA-enhanced induction are those of the germination program rather than postabscission program.

The second difference in hormone response also involves germination mRNAs. Unlike in younger embryos, GA does not enhance significantly the expression of germination mRNAs in cultured predesiccation-stage or mature embryos (Figures 1E and 1F; Figure 2, compare lanes 11 and 13; other data not shown). A trivial reason for this difference would be that older embryos no longer respond at a molecular level to exogenous GA. However, internal evidence shows that at least mature embryos still respond to the hormone. GA in GA + ABA medium abolishes the ABA-dependent maintenance or reaccumulation of *Mat*, *MatP*, *Lea*, and *LeaA* mRNAs and reduces significantly the ABA enhancement of *Grm* and *GrmMP* mRNAs, although it does not rescue ABA suppression of germination mRNAs (data not shown).

Precocious Desiccation Induces *Lea* and *LeaA* mRNAs and Then Turns Off the Postabscission Program

Cotyledons of 2 hr-imbibed embryos older than 26 DPA survive in vitro desiccation when applied over a 1.5-day period, and in subsequent culture they germinate as rapidly as 50 DPA to 53 DPA predesiccation-stage embryos and mature dry embryos (data not shown). All 18 *Lea* and *LeaA* mRNAs accumulate very rapidly in 29 DPA and 40 DPA cotyledons during such a desiccation of maturation-stage embryos (Figures 1B and 1C, open diamonds; Figure 2, compare lanes 8 with lanes 1). This accumulation is faster than the one that occurs during direct culture for a comparable length of time (Figures 1B and 1C; Figure 2, compare lanes 8 with lanes 2 and 3). However, the desiccation protocol does not change greatly the initial levels of the maturation-specific and germination-specific mRNAs that, like *Lea* and *LeaA* mRNAs, also change rapidly in concentration during direct culture (Figures 1A, 1E, and 1F; Figure 2, compare lanes 8 with lanes 2 and 3). Thus, we conclude that during these experiments most of the increase in *Lea* and *LeaA* mRNAs over their initial levels is due specifically to desiccation.

There is little evidence in these experiments for desiccation-specific induction of mRNAs of other classes that are expressed in the postabscission program, in particular of *MatP* and *GrmCP* mRNAs (Figure 1D; Figure 2, lanes 8), in which such an induction might be detected over possible imbibition-associated changes in the initial levels of the mRNAs. We do not view these as conclusive results, however, because their kinetics were not measured in these experiments.

As noted earlier, desiccation normally occurs after 53 DPA and thus cannot terminate the postabscission program in the plant that occurs at 50 DPA. However, precocious desiccation is the only treatment tried so far that terminates its expression in cultured immature embryos. *Lea*, *LeaA*, and *MatP* mRNAs disappear during culture of precociously desiccated embryos at rates close to those in cultured 50 DPA to 53 DPA predesiccation-stage embryos or mature embryos (Figures 1B, 1C, and 1D, closed diamonds; Figure 2, compare lanes 8 with lanes 11 and 15). That these mRNA losses are not caused by desiccation damage is evidenced in particular by the three germination-specific *Grm* D125, *GrmC* D142, and *GrmP* E41 mRNAs and the three *GrmM* mRNAs in the same embryos. As during direct culture of excised embryos, their levels increase rapidly in precociously desiccated embryos upon their rehydration and culture (Figures 1E and 1F; Figure 2).

DISCUSSION

Those mRNAs that are expressed coordinately in normal development are expressed coordinately under a wide variety of culture conditions in late cotyledon-stage to postabscission-stage embryos. This result allows the assignment of gene expression in culture to execution of the developmental programs earlier inferred to exist during normal development (Hughes and Galau, 1989). It also suggests that the component mRNAs in the individual programs are regulated coordinately in both situations. Furthermore, their expression in culture and in the plant is explicable readily in terms of global effects of a small number of events that occur during development.

Simultaneous Induction of Postabscission and Germination Programs in Excised Maturation-Stage Embryos

At least by the late cotyledon stage, embryos are competent to express both postabscission and germination programs in culture but do not do so in the plant. These results are in contrast to earlier ones that depict precociously germinating immature cotton embryos as largely similar to germinating mature embryos (Dure et al., 1981). Interpretations of gene activity in cotton and other species

often involve dual alternative pathways for excised embryos (Dure et al., 1981; Quatrano et al., 1983; Finkelstein et al., 1985; Sánchez-Martínez et al., 1986; reviewed by Crouch, 1987). When precociously germinating, they leap ahead onto the germination path, but if cultured on ABA (or OSM), they are inhibited from germinating and continue as maturation-stage embryos or jump ahead onto what is now recognized as the postabscission path. Our results show definitively that both postabscission and germination paths are traversed simultaneously on basal medium, whereas the maturation path is not.

Excision and culture are equivalent experimentally to ovule abscission in the plant in their induction of the postabscission program but not in their induction of the germination program. We predict that excision is sufficient for induction of the postabscission program, whereas induction of the germination program at least requires imbibition, if not excision. Excision removes potential mechanical restrictions and the source of potential maternal influences on immature embryos, whereas subsequent culture additionally leads to significant increases in water content and the possible dilution of any regulatory substances. Because there is no observable change in mechanical restraint or embryo water content during expression of the postabscission program, the relevant part of the culture process that mimics in-plant conditions at 45 DPA is probably the excision itself. In support of this conclusion, culture of embryos on basal medium cannot restore the postabscission program at 50 DPA to 53 DPA (Figures 1B and 1C; Figure 2) or greatly affect the program once it is induced in the plant at 46 DPA to 48 DPA (data not shown). In addition, the composition of the culture medium does not alter greatly the rate or extent of the changes in the maturation and postabscission programs (data not shown).

The Maturation Program in Excised Embryos

Although all five *Mat* mRNAs in maturation-stage embryos decline in culture with initial rates to levels very similar to those achieved after ovule abscission, four *Mat* mRNAs then increase twofold to 20-fold before apparently continuing to decline at a lower rate. This may indicate that the maturation program is expressed in excised and cultured maturation-stage embryos, but transiently and at a very low level of less than 10^{-2} the level in the plant. However, it is more likely that this increase is due to expression under the postabscission program. That is, these mRNAs are really *MatP* mRNAs but with postabscission components so minor that they are obvious only by their behavior in cultured embryos. This interpretation is consistent with the minor enhancements of their levels by ABA in cultured embryos of all ages because a minor postabscission component should have the same response to ABA as the postabscission-abundant *Lea*, *LeaA*, and, most significantly, *MatP* mRNAs that have obvious postabscission components during normal development.

Normal and Precocious Desiccation and Termination of the Postabscission Program

One view of the regulation of embryogenesis is that normal embryo desiccation or experimental desiccation of immature excised embryos is required to switch from embryogenic to germination programs (Kermode and Bewley, 1986). Two of our results do not support this model: the germination program expressed in excised embryos does not require prior desiccation of the embryo, and both the maturation and the postabscission programs are terminated in the plant before desiccation starts. However, our results do support two aspects of the model: before its termination in the plant, the postabscission program (but never the maturation program) is always expressed in excised embryos, and precocious desiccation is the only treatment so far tried that can turn it off, if only after specifically enhancing its induction.

There are two quite different ways to reconcile the termination of the postabscission program in the plant before desiccation with its termination upon culture after precocious desiccation. The first is that some kind of nonobvious, incipient desiccation is the normal termination signal in the plant and that it is mimicked by the precocious desiccation protocol. However, if completion of the postabscission program is essential for survival of the embryo during desiccation and water stress (see below), incipient desiccation should be an inducing signal rather than the termination signal. We favor a second explanation, that the postabscission program is terminated by a critical concentration of one or more of its encoded proteins. With such a mechanism, developmentally programmed desiccation would begin only upon completion of the protective program in the plant, and precocious desiccation protocols that result in sufficient accumulation of protein products of the induced postabscission program should, upon subsequent culture, terminate the program.

Termination of Postabscission mRNA Expression and Improved Quality of Germination

Precociously desiccated immature embryos, predesiccation-stage 50 DPA to 53 DPA embryos, and normal dry, mature embryos all have high levels of *MatP*, *Lea*, and *LeaA* mRNAs before culture but rapidly lose them upon culture (Figures 1B, 1C, and 1D; Figure 2). The rate and quality of germination and seedling growth of all three kinds of embryos are also much superior to that observed

in directly cultured maturation-stage or postabscission-stage embryos (data not shown) that induce and/or maintain the postabscission mRNAs (Figures 1B, 1C, and 1D; Figure 2). This correlation suggests that continued expression of the postabscission program in directly cultured young embryos inhibits embryo germination and seedling growth but apparently not greatly the expression of many germination-abundant mRNAs. Improved germination and/or seedling growth has been observed in several species of immature embryos if their imbibition is delayed for several days after excision, especially if a very modest water loss is imposed during the delay (cf. Rosenberg and Rinne, 1986; Bewley et al., 1989). In soybean, at least, during this time there is accumulation of proteins unique to late embryogenesis (Rosenberg and Rinne, 1988) that are probably the proteins encoded by *Lea* and *LeaA* of the postabscission program. Although we have not used these protocols, the results presented here predict that the postabscission mRNAs are induced by excision, and the maintenance of the embryo at near its in-plant water potential allows completion of the postabscission program and the subsequent full ability to sustain seedling growth upon imbibition.

Regulation of Gene Expression during Normal Development and Culture

Our results are consistent with the hypothesis that a maternal maturation factor sustains the maturation program in the plant (Dure et al., 1981; Galau et al., 1987; Hughes and Galau, 1989). *Mat* mRNAs are not maintained significantly in cultured maturation-stage embryos by any method tried, including ABA, GA, OSM, or combinations of these (Figure 1A; Figure 2; data not shown), and the very minor modulation of some of these *Mat* mRNAs by ABA and OSM can be attributed very reasonably to their very minor expression under the postabscission program. The conclusion is that a regulatory factor must be present in the embryo that is lost rapidly upon abscission or in culture after excision. During maturation, this factor probably suppresses the postabscission and germination programs in embryos that are otherwise competent to express them.

We suggest that the maturation factor inhibits accumulation of a positively acting, endogenous postabscission factor that is required for induction of postabscission mRNAs. Alternative circuits are possible, but this version explains best the expression of *MatP* mRNAs, which appear to be controlled positively by both the maturation factor and the abscission-associated and excision-associated release from that factor exemplified by *Lea* and *LeaA* mRNAs. Induction of the germination program requires a factor that is present in culture but not in postabscission embryos in the plant. Inhibition of water is the obvious candidate.

Exogenous ABA or GA Cannot Drive Normal Development in Cultured Embryos

The effects of ABA, OSM, and GA on gene expression in cultured immature embryos appear to be dominated by that induced by the excision and culture events themselves. ABA only enhances expression of the induced postabscission program and reduces expression of the induced germination program. Conversely, GA only enhances expression of the germination program and reduces expression of the postabscission program. Although their nearly reciprocal effects are similar to those observed in other systems (cf. Ho and Varner, 1976; Jacobsen and Beach, 1985; Lin and Ho, 1986), and each accentuates one of the two developmental programs, we conclude that simple removal of the embryo from the maternal environment and its increase in water content during culture both cause profound changes that cannot be abolished but only accentuated differentially by exogenous growth regulators.

We reject the notion that there is a biologically significant accumulation of ABA in embryos cultured on basal medium and that this, rather than excision, is the inducer of the postabscission program. Endogenous ABA declines rapidly during embryo culture (Table 1), as it does in other cultured dicot embryos (Ackerson, 1984; Bray and Beachy, 1985; Finkelstein et al., 1985), and the induction of the postabscission program is not sensitive to the ABA synthesis inhibitor fluridone (Table 2). The decline in ABA is not altered significantly in embryos cultured on OSM, which enhances the level of *Lea* and *LeaA* mRNAs approximately the same as that achieved with 1 μM to 5 μM ABA (Table 1). Furthermore, if we can attribute the reciprocal effects of exogenous ABA and GA on particular mRNAs to increased active levels of ABA and GA in the embryos, then these or similar-acting hormones cannot accumulate to significant levels in embryos on basal medium.

Although ABA has often been considered an endogenous maturation or postabscission factor affecting gene activity (Dure et al., 1981; Bray and Beachy, 1985; Finkelstein et al., 1985; Quatrano, 1986), our results do not support such a role for ABA during either period. The termination of the maturation program in cultured maturation-stage embryos is affected little, if at all, by exogenous ABA even though the level of ABA in dicots is highest in late cotyledon-stage and early maturation-stage embryos that have induced the maturation program in the plant (Hsu, 1979; Koornneef et al., 1982; Ackerson, 1984; Finkelstein et al., 1985; Galau et al., 1987). Only the early expression of *LeaA* mRNAs is at all correlated with the level of endogenous ABA (Galau et al., 1987; Hughes and Galau, 1989), but their expression in culture is identical to

that of *Lea* mRNAs that do not have an obvious ABA-associated component. The consistent induction of the postabscission mRNAs in embryos of virtually any age on basal medium also argues very strongly against endogenous ABA having any role in the induction or maintenance of the postabscission program in the plant. We conclude that the only way endogenous ABA could be responsible for induction of the postabscission program both in the plant and in culture would be for it to be in a form or location during postabscission that is not obvious in bulk measurements (Zeevaart and Creelman, 1988) and that its endogenous activity in maturation-stage embryos would have to be increased vastly upon their culture. A much simpler alternative is that it plays no significant role in the induction of the postabscission program in either context.

OSM, Exogenous ABA, and Desiccation as Environmental Inducers of the Postabscission Program

The major function of the postabscission program has been proposed to be protection during desiccation and repair of desiccation-induced damage (Galau et al., 1987; Hughes and Galau, 1989). The amino acid sequences of several cotton proteins encoded by *Lea* and *LeaA* (Baker et al., 1988) and related proteins in other species (see Dure et al., 1989; Skriver and Mundy, 1990) have been interpreted as suggesting a water stress-protective or desiccation-protective function (Baker et al., 1988; Dure et al., 1989). The results presented here demonstrate clearly that culture of maturation-stage embryos on OSM or exogenous ABA enhances the expression of the entire postabscission program and precocious desiccation induces at least the *Lea* and *LeaA* components of the postabscission program. Other experiments show that the entire cotton postabscission program, including *MatP* mRNAs, can be induced as late as 2 days into normal germination by ABA, OSM, and desiccation (M. Swain and G.A. Galau, unpublished data). In more limited experiments in monocot species, at least some *Lea*-like mRNAs can be maintained or reinduced in germinating mature embryos by ABA (Mundy and Chua, 1988; Williamson and Quatrano, 1988) or osmotic stress (Morris et al., 1990) and in vegetative organs by a variety of stresses (reviewed in Skriver and Mundy, 1990).

A simple unifying hypothesis is that the postabscission program has at least two inducers. The first is a developmental one, associated with ovule abscission, that initiates preparation for forthcoming desiccation. As seems perfectly reasonable, ABA, water stress, or desiccation have nothing to do with its induction, maintenance, or termination in the plant. The second inductive system is associated with water stress and is significant normally during early germination in a variable environment where maintenance of postabscission proteins through environmental reinduction of the program could be protective during water stress or redesiccation. As demonstrated by their induction of germination mRNAs on basal medium, cultured maturation-stage embryos germinate at the molecular level, and, consequently, they should be able to respond as germinating embryos to germination-associated water stress by inducing the postabscission program, even though the program is also induced by the developmentally relevant excision event. It is this environmental enhancement of a developmental program that has been nearly universally misunderstood as indicative of its developmental regulation.

METHODS

Embryo Culture

Gossypium hirsutum cv Coker 201 was maintained in a greenhouse. For most experiments, all embryos in parallel culture treatments were from a single boll. Representative embryos were staged (Galau et al., 1987), and sibling embryos were prepared and cultured in the dark at 28°C under sterile conditions on a basal medium composed of PG inorganic salts, 1% sucrose, and 0.5% agarose (Galau et al., 1986). Where indicated, autoclaved basal medium was supplemented with 1 μM to 10 μM GA, 0.025 μM to 5 μM ABA (0.1 μM to 20 μM mixed isomers, Sigma Chemical Co.), or 0.3 μM to 30 μM fluridone (technical grade, Eli Lilly) by the addition of ethanol (fluridone) or filter-sterile aqueous (GA, ABA) stock solutions. OSM was basal medium autoclaved with 6% to 12% mannitol. Before culture, embryos were washed for 1 hr two times in water or in water containing additives that were present during their subsequent culture. Mature embryos were treated similarly after removal from delinted seeds that were sterilized in 20% commercial bleach and rinsed in water.

For experimental desiccation, washed maturation-stage embryos were placed over Drierite in Petri dishes with unsealed lids. Water loss was 2.5% of the washed embryo initial wet weight/hour; percentage water declined from 80% to 65% by 20 hr and to <10% by 36 hr. Before culture on basal medium, the dry embryos were imbibed for 1 hr in water and washed for 0.5 hr in water.

Measurements of Endogenous ABA

Nonbound ABA (defined here as the [+] *cis-trans* isomer) was measured by semicompetitive enzyme-linked immunosorbent assay using the C-5 monoclonal antibody of Mertens et al. (1983) as described (Galau et al., 1987; Raikhel et al., 1987). Uncertainties of the measurements are approximately ±10% (Galau et al., 1987).

Analysis of mRNAs

Total RNA was prepared from cotyledons of one to three embryos, as described previously (Hughes and Galau, 1989), and 1 μg was analyzed by formaldehyde-gel electrophoresis (Galau et al., 1986) and/or RNA dot blots (Galau et al., 1987). RNA transfers were onto GeneScreen membranes (Du Pont-New England Nuclear). The membranes were then washed, air dried, irradiated with UV light, and heated for 1 hr at 80°C under vacuum (Galau et al., 1986). They were hybridized at Tm 15°C (Galau et al., 1983, 1986) with ^{32}P-labeled cloned cDNA inserts described earlier (Hughes and Galau, 1989). With the exception of the vicilin probe *Mat* C72, all cDNA probes hybridize at this criterion with only the two very similar alloalleles in the *G. hirsutum* tetraploid genome (Galau et al., 1988; Hughes and Galau, 1989). Estimates of mRNA abundance, expressed as a relative fraction of total RNA, were made from autoradiograms of the RNA dot blots (Galau et al., 1987; Hughes and Galau, 1989). Their concentrations as a percentage of total RNA may be calculated from the data of Galau et al. (1987) and Hughes and Galau (1989).

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