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Gibberellins and Seed Development in Maize. II. Gibberellin Synthesis Inhibition Enhances Abscisic Acid Signaling in Cultured Embryos[1]

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Abscisic acid (ABA) is required for seed maturation in maize (*Zea mays* L.) and other plants. Gibberellins (GAs) are also present in developing maize embryos, and mutual antagonism of GAs and ABA appears to govern the choice between precocious germination or quiescence and maturation. Exogenous ABA can also induce quiescence and maturation in immature maize embryos in culture. To examine the role of GAs versus ABA in regulating maize embryo maturation, the effects of modulating GA levels were compared with those of ABA in embryos cultured at successive stages of development. The effects of GA synthesis inhibition or exogenous GA application differed markedly in embryos at different stages of development, indicating changes in both endogenous GA levels and in the capacity for GA synthesis as embryogenesis and maturation progress. In immature embryos, the inhibition of GA synthesis mimicked the effects of exogenous ABA, as shown by the suppression of germination, the acquisition of anthocyanin pigments, and the accumulation of a variety of maturation-phase mRNAs. We suggest that GA antagonizes ABA signaling in developing maize embryos, and that the changing hormone balance provides temporal control over the maturation phase.

The phytohormone abscisic acid (ABA) plays a variety of roles in plant growth and environmental response, including a critical role in the development of seeds. A rise in the ABA level during embryogenesis triggers processes that lead to the acquisition of desiccation tolerance and seed dormancy. The role of ABA in establishing these characters has been demonstrated by the behavior of mutants that disrupt ABA synthesis or alter ABA sensitivity. In Arabidopsis, mutants that are deficient in ABA synthesis and mutants that are ABA-insensitive show reduced seed maturation and dormancy (Koornneef and Karssen, 1994; Leon-Kloosterziel et al., 1996). In maize (*Zea mays* L.), many seed-specific ABA responses are due to increases in gene expression that are synergistically regulated by ABA and the transcription factor VP1 (McCarty et al., 1991; Hattori et al., 1992). Mutant maize embryos that are deficient in ABA synthesis or lack an active VP1 factor do not express the normal suite of maturation phase proteins and they do not acquire desiccation tolerance. Unlike in Arabidopsis, these mutants do not become quiescent but germinate precociously on the ear.

The timing of *Vp*1 gene expression and the flux of ABA levels during seed development are not perfectly correlated with the timing of ABA-induced maturation events, suggesting that other modulating factors are also relevant. For example, the induction of the LEA (late embryogenesis abundant protein) genes that are thought to function in desiccation tolerance occurs much later than the time of highest endogenous ABA levels in cotton and maize, although these genes have been shown to require ABA for expression (Galau et al., 1987; Thomann et al., 1992; White and Rivin, 1995). This paradox also extends to the germination behavior of immature maize embryos. Embryos excised in the early maturation phase (when endogenous ABA levels are known to be high) germinate readily when cultured with basal medium, while late maturation stage embryos, which have diminished ABA levels, are nonetheless quiescent in culture (Rivin and Grudt, 1991).

In the accompanying paper (White et al., 2000), we show that gibberellins (GAs) are another constituent that needs to be considered in understanding the temporal organization of maturation phase regulation in maize. Bioactive GA species accumulate early in maize embryogenesis and decline in concentration as embryo ABA levels peak. A similar relationship of active GA and ABA peaks has been found in barley and wheat embryos (Jacobsen et al., 1995). We found that inhibiting GA synthesis during the period of GA accumulation (embryo stage 2) could suppress the vivipary of ABA-deficient *vp*5 mutant kernels, while inhibition of GA synthesis later in embryo development had no effect on vivipary. These data suggest that the rise in embryo ABA in mid development acts to repress a positive GA signal for germination. Thus, GA and ABA antagonism controls a decision point between vivipary versus quiescence that occurs at or before stage 2 of embryo development. In contrast, the VP1 component of quiescence regulation appears to be independent of this early GA-ABA antagonism, as inhibition of GA synthesis had no pronounced effect on the behavior of *vp*1 mutant kernels.

The germination behavior of the quiescent ABA-deficient (*vp*5) kernels created by GA synthesis inhibition suggested a further early role for GAs in modulating maturation

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phase processes. The quiescent mutant seeds were found to germinate comparably to their wild-type sibling kernels after drying and prolonged dry storage. Desiccation tolerance and storage longevity have been associated with ABA/VP1-induced processes, including the accumulation of maturation phase proteins (for review, see Bewley, 1997). These results imply that GA levels also influence ABA-controlled gene expression, possibly by affecting ABA sensitivity. When endogenous kernel GA levels are depressed during ABA-deficient embryo development, the small maternal ABA component (Smith et al., 1987) is sufficient to induce a complete maturation program, including the expression of maturation-specific genes.

To further analyze the possible roles of GAs in prematuration and maturation-phase regulation, we examined the germination behavior and gene expression of isolated maize embryos. Cultured plant embryos have been widely used to model physiological cues for maturation. Isolated embryos of maize and many other species have been shown to suppress germination and either initiate or maintain expression of maturation-associated genes when cultured in the presence of exogenous ABA (for review, see Skriver and Mundy, 1990). The possible involvement of the GAs in modulating seed maturation has been much less fully examined, although there a few reports of exogenous GA treatment down-regulating maturation-associated gene expression in culture (Leah and Mundy, 1989; Bartels et al., 1991; Hughes and Galau, 1991). In these studies we compared how embryos at successive stages of development responded to the modulation of GA levels. We found that chemical inhibition of GA synthesis in immature cultured embryos mimicked the effects of exogenous ABA application in the suppression of germination, the accumulation of anthocyanin pigments, and the expression of a variety of maturation-phase genes. However, GA synthesis inhibition did not change the requirement for, or the expression of the *Vp*1 gene. These results indicate that GAs present in immature maize embryos antagonize ABA in regulating both embryo quiescence and gene expression, and so play a role in organizing the maturation program in maize.

MATERIALS AND METHODS

Plant Material

The inbred line W22 used in this research was originally obtained from J. Kermicle (University of Wisconsin, Madison). It carries the *R*-sc allele. The maize (*Zea mays* L.) viviparous mutants *vp*5, *vp*1, *vp*1-McW, and source material for the *R*-g and *R*-r alleles were obtained originally from D. Robertson (Iowa State University, Ames). All stocks were propagated in our maize nursery in Corvallis, Oregon. Homozygous *vp*5 kernels were identified on segregating ears by their lack of carotenoid pigments. These mutant kernels showed 100% precocious germination by stage 4 of development. Kernels homozygous for the *vp*1 allele were obtained from homozygous mutant plants propagated from precociously germinating seeds, or were identified on segregating ears by the absence of anthocyanin-pigmented aleurone.

Embryo Culture

Plants were grown in the field and ears were harvested between 18 and 30 d after pollination (DAP) and surface-sterilized in 2.5% (w/v) sodium hypochlorite. Embryos were removed aseptically and staged morphologically by examining leaf primordia development according to the scheme of Abbe and Stein (1954). Stages 2, 3, and 4 of embryo development typically corresponded to 18 to 22, 23 to 27, and 28 to 30 DAP, respectively. For culture in Petri dishes, 10 to 25 embryos were placed scutella down on filters saturated with a growth medium consisting of Murashige and Skoog medium (Sigma, St. Louis) supplemented with 3% (w/v) Suc, 100 mg/L myoinositol, and 0.4 mg/L thiamine HCl plus growth regulators where noted. ABA (mixed enantiomers), ancymidol, and GA_3 were purchased from Sigma. Paclobutrazol obtained from ICI was the gift of William Proebsting (Oregon State University). Cultures were incubated at 26°C in the dark. Germination was scored daily based on the criterion of radicle emergence from the coleorhiza. Unless otherwise noted, data were pooled from replicate experiments and values are given as means ± SE.

Anthocyanin Determinations

Anthocyanins were extracted from embryos with acidified methanol, and absorbance was determined at 530 nm in the linear range (Mancinelli et al., 1988).

Northern Blots

Total RNA was prepared from maize embryos using slight modifications of the Chomczynski and Sacchi method (1987). Northern analysis was performed using low formaldehyde (2%, w/v), 1.2% (w/v) agarose, and 3-(N-morpholino)-propanesulfonic acid (MOPS) gel electrophoresis. Gel loading was determined by staining duplicate blots with methylene blue. Hybridizations were done overnight at 68°C in 250 mM NaH_2PO_4, 7% (w/v) SDS, and 100 μg/mL salmon sperm DNA using random hexamer-^{32}P labeled probes and standard washing conditions. Some of the cDNA clones used as probes were isolated from a Lambda Zap II (Stratagene, La Jolla, CA) library prepared from maturation phase maize embryos (White, 1995; White and Rivin 1995a, 1995b). These correspond to the following proteins: GLB1, an embryo storage protein (Belanger and Kriz, 1991); MEC, a metallothionein protein homologous to wheat Ec (White and Rivin, 1995a); the LEA protein MLG3 (maize LEA group 3) (White and Rivin, 1995b); MEM, the maize homolog to the wheat Met-rich protein Em (White, 1995). The cDNA clones for the LEA protein dehydrin (DHN) (Close et al., 1989) and the transcriptional activator protein VP1 (McCarty et al., 1991) were provided by Drs. Timothy Close (University of California, Riverside) and Donald McCarty (University of Florida, Gainesville).

RESULTS

Maturing Embryos Require GA for Germination in Culture

Wild-Type Embryos

Wild-type maize embryos germinate when they are removed from the seed and placed in a hormone-free culture medium. The rate of germination and the percentage of embryos that ultimately germinate is different for each developmental stage (Rivin and Grudt, 1991). From the previous experiments with viviparous mutants, we hypothesized that active GA is required for the germination of isolated embryos, and that a balance between GA and ABA levels would determine the propensity of embryos to germinate in conditions in which water is not a limiting factor (White et al., 2000). To determine whether the germination of immature embryos requires GA synthesis, we tested the effect of two GA synthesis inhibitors, paclobutrazol and ancymidol, on isolated embryos cultured at successive stages of development. The embryos were dissected from several sib-pollinated ears at various times during stages 2, 3, and 4 corresponding to the pre-, early-, mid-, and late-maturation phases. Samples of staged embryos from several ears were placed in GM (a hormone free growth medium), in GM plus 10 or 100 μM ancymidol, in GM plus 10 or 100 μM paclobutrazol, and in GM plus 10 μM ABA. Germination was measured daily for 8 d. The results are shown in Figure 1.

Maize embryos at successive stages of development show distinctive germination patterns on hormone-free medium. Stage 2 embryos (pre-maturation phase) and stage 3 embryos (early and mid-maturation phase) germinate well after a short lag. In contrast, stage 4 embryos (late maturation phase) were nearly quiescent when cultured on GM alone. Germination of all these stages was suppressed when ABA was added to the medium (Fig. 1, A, D, G, and J).

Treatment with GA synthesis inhibitors also decreased both the rate of germination and the fraction of embryos that germinate, but these effects were found to be contingent on the developmental stage. The application of ancymidol (Fig. 1, B, E, H, and K) or paclobutrazol (Fig. 1, C, F, I, and L) strongly suppressed the germination of both

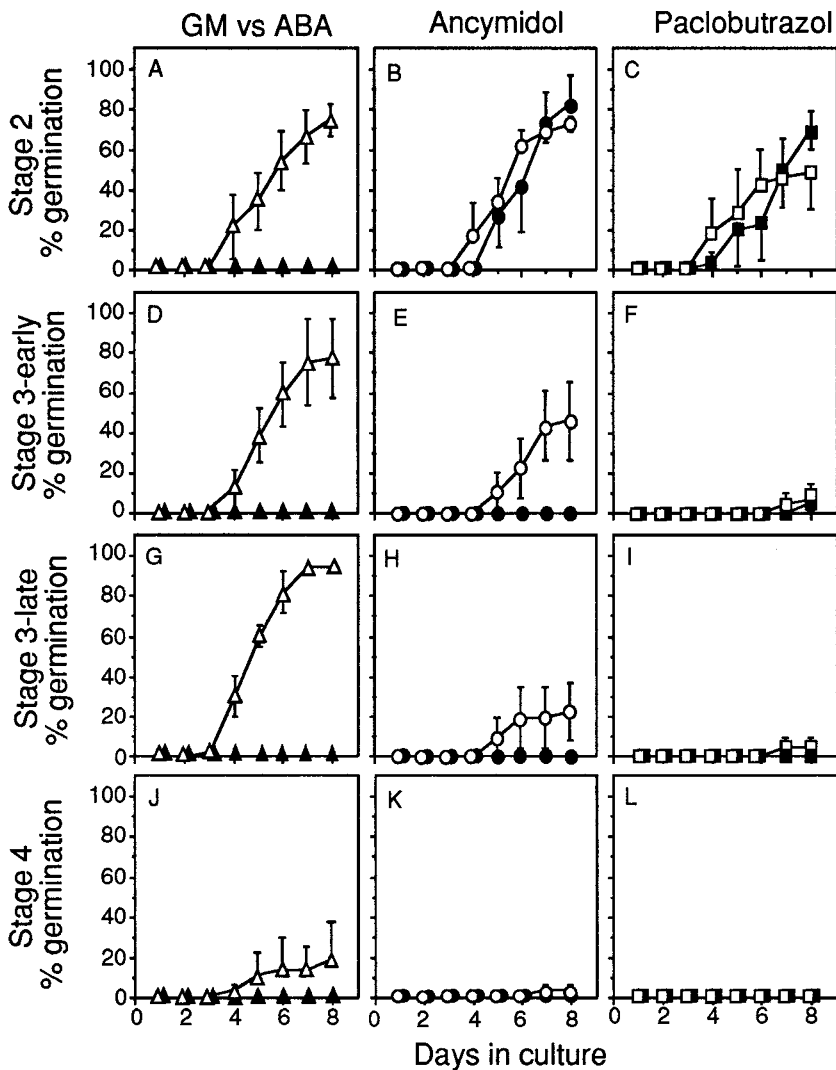

Figure 1. Germination of wild-type embryos isolated at successive developmental stages when cultured in the presence and absence of growth regulators and inhibitors. A through C, Stage 2 embryos; D through F, early stage 3 embryos; G through I, late stage 3 embryos; J through L, stage 4 embryos. Cultures were maintained for 8 d on hormone-free GM (△) or on GM supplemented with 10 μM ABA (▲), 10 μM ancymidol (○), 100 μM ancymidol (●), 10 μM paclobutrazol (□), or 100 μM paclobutrazol (■). Where required for clarity, error bars are shown in only one direction.

sets of stage 3 embryos and embryos at stage 4. However, these treatments had little effect on stage 2 embryos.

To learn if the effects of ancymidol or paclobutrazol are mediated by a reduction in GA levels, we tested whether the addition of exogenous GA would restore germination to inhibitor-treated stage 3 and stage 4 embryos. The embryos were cultured in 100 μM paclobutrazol with and without the addition of GA_3. As shown in Figure 2, the hormone treatment restored the germination levels of stage 3 embryos to that of embryos cultured on GM alone, indicating that paclobutrazol suppressed the germination of these embryos by inhibiting de novo GA biosynthesis. Similar results were obtained with ancymidol cultures (data not shown).

Exogenous active GA also had a pronounced effect on the germination of stage 3 and stage 4 embryos in the absence of GA synthesis inhibitors (Fig. 3). The addition of GA_3 to stage 3 cultured embryos decreased the lag time before germination compared with embryos in GM alone. Strikingly, exogenous GA_3 treatment caused rapid and complete germination of stage 4 embryos, which were quiescent in hormone-free medium.

vp1 Mutants

In experiments in vivo, the vivipary of *vp*1 mutants was not significantly effected by treatment with GA synthesis inhibitors or by double mutant combinations with GA-deficient dwarfs (White et al., 2000). Therefore, it was of interest to determine whether *vp*1 mutant embryos require new GA synthesis to germinate in culture. Two types of *vp*1 mutants were tested: a standard allele that makes no active VP1 protein and *vp*1-McW, an allele that produces a truncated product (Carson et al., 1997). Seeds homozygous for the standard allele are viviparous, while *vp*1-McW seeds are quiescent on the ear.

Figure 4 shows the germination behavior of stage 4 *vp*1-null and *vp*1-McW embryos in hormone-free GM medium, in paclobutrazol, and with exogenous ABA. Unexpectedly,

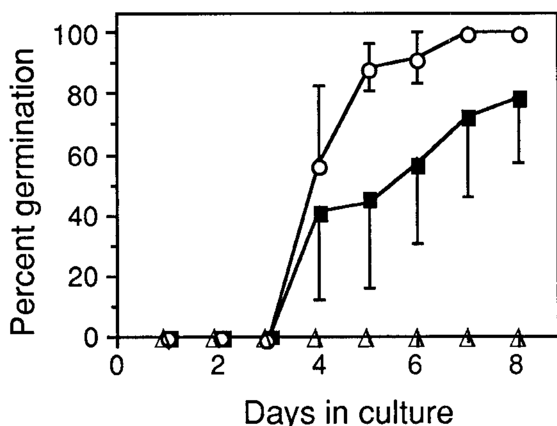

Figure 2. Effects of exogenous GA_3 on the germination of paclobutrazol-treated embryos. Wild-type embryos at late stage 3 were isolated and cultured for 8 d in GM plus 100 μM paclobutrazol alone (△) and with either 10 μM GA_3 (■) or 100 μM GA_3 (○).

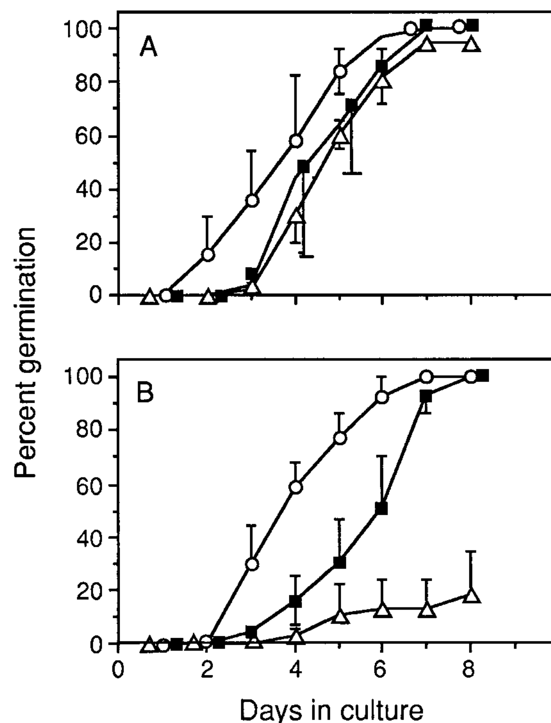

Figure 3. Effects of exogenous GA_3 on the germination of wild-type embryos in culture. Wild-type embryos at late stage 3 (A) or stage 4 (B) were cultured for 8 d on GM alone (△) or on GM supplemented with 10 μM GA_3 (■) or 100 μM GA_3 (○).

the behavior of the quiescent *vp*1-McW mutant was very similar to that of the viviparous *vp*1-null mutant, and quite different from that of their wild-type sibling embryos. The isolated *vp*1-null allele mutants germinated almost immediately upon excision and were affected very little by paclobutrazol treatment. They also showed no inhibition by ABA, as expected. After a brief lag, the stage 4 *vp*1-McW embryos also germinated rapidly. Unlike the viviparous allele, *vp*1-McW embryos displayed some sensitivity to both the GA synthesis inhibitor and to ABA. These embryos were not ultimately suppressed, but they showed a substantial delay in germination. Shoot elongation rates were severely reduced in all of the paclobutrazol cultures, indicating that mutants and wild types were sensitive to GA levels in this aspect of their growth (data not shown).

Developmentally Regulated Anthocyanin Synthesis in Cultured Embryos Responds to ABA and GA

Another embryo character governed by ABA and VP1 is the accumulation of anthocyanin pigments. Anthocyanin synthesis in the embryos is activated by transcriptional regulators encoded by the *C*1 and *R* genes (Roth et al., 1991). *C*1 expression is regulated synergistically by ABA and the *Vp*1 gene product (Hattori et al., 1992). Many *R* alleles exist, but only a few are expressed in embryo tissue. On the ear, embryos that carry the *R*-sc allele begin to accumulate significant anthocyanin pigments in early stage 4. We tested whether developmentally specific anthocya-

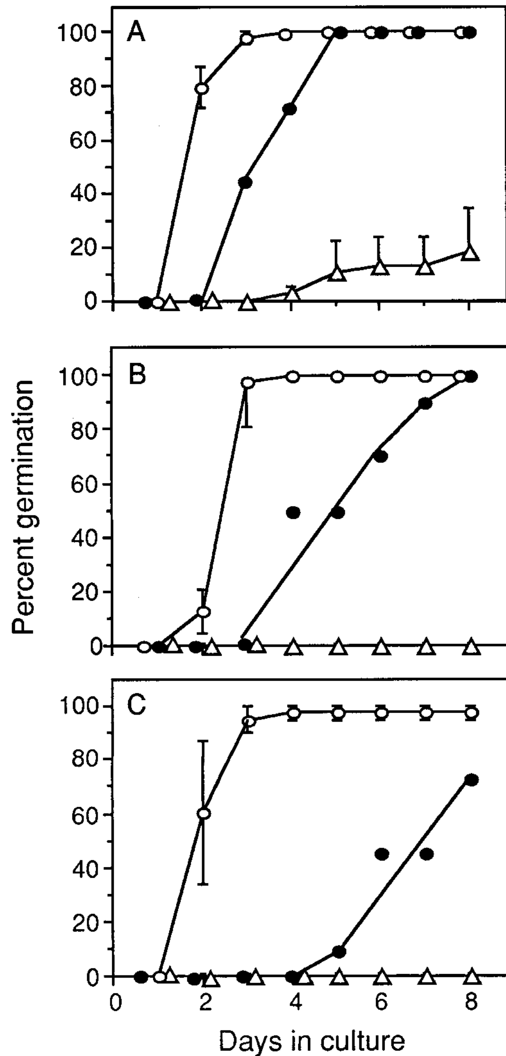

Figure 4. Germination of cultured embryos homozygous for *vp*1 (viviparous) or *vp*1-McW (dormant) alleles. Wild-type embryos (△) and embryos homozygous for either *vp*1 (○) or *vp*1-McW (●) were isolated at stage 4 and cultured for 8 d in GM (A), 100 μM paclobutrazol (B), or 10 μM ABA (C). Similar results were obtained from stage 3 *vp*1-McW embryos cultured under parallel conditions.

nin accumulation in cultured embryos was influenced by modulation of GA and ABA.

When isolated stage 4 *R*-sc embryos were cultured in GM alone, they showed considerable anthocyanin accumulation. The level of pigmentation was increased in embryos cultured with either paclobutrazol or 10 μM ABA. Pigmentation was severely diminished when paclobutrazol-treated cultures were supplemented with exogenous GA_3 (Fig. 5). Precocious pigmentation could be induced in younger embryos by culturing them in the presence of 10 μM ABA (Fig. 6A). As in the mature embryos, the addition of paclobutrazol to the medium resulted in a level of anthocyanin accumulation equivalent to that induced by ABA, and this could be obviated by the addition of active GA (Fig. 6B).

Anthocyanins accumulate in a developmentally and tissue-specific manner, but can also be synthesized in response to stress. To correlate the regulation of anthocyanin accumulation in cultured embryos with developmentally programmed pigment accumulation, we tested whether the synthesis of anthocyanins in culture was dependent on the appropriate *R* allele. Embryos carrying the *R*-r or *R*-g alleles did not accumulate anthocyanins as part of their maturation program in vivo. When embryos of these genotypes were cultured in the presence of exogenous ABA or GA synthesis inhibitors, they did not accumulate anthocyanin pigment (Fig. 6C).

GA Biosynthesis Inhibition Stimulates the Precocious Accumulation of Maturation Phase mRNAs in Cultured Embryos

The effect of modulating GA content in cultured embryos was monitored at the level of gene expression by measuring the steady-state mRNA levels of genes normally

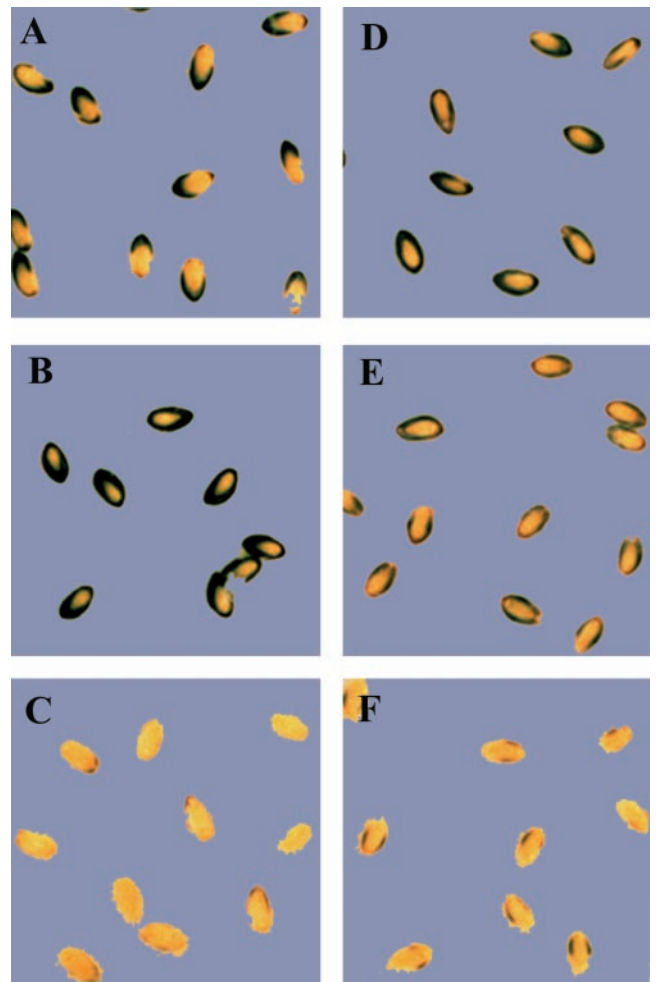

Figure 5. Anthocyanin accumulation in cultured embryos. Stage 4 embryos were cultured for 1 d in GM (A) or in GM supplemented with 10 μM ABA (B), 10 μM GA_3 (C), 100 μM paclobutrazol (D), 100 μM paclobutrazol plus 10 μM GA_3 (E), or 100 μM paclobutrazol plus 100 μM GA_3 (F).

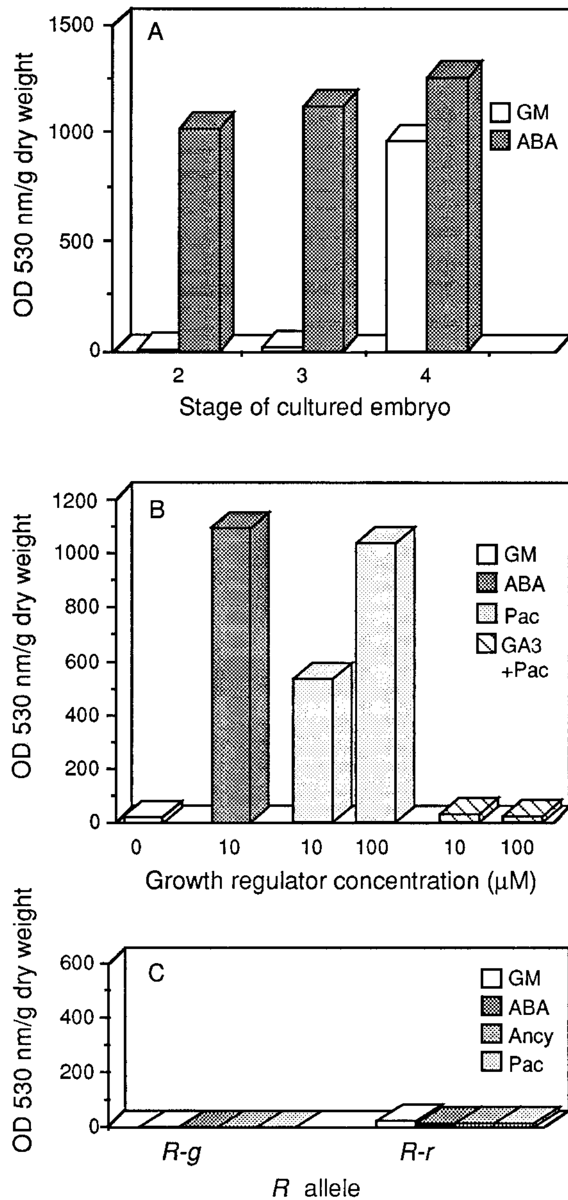

Figure 6. Developmental specificity and hormonal regulation of anthocyanin accumulation in cultured embryos. A, Anthocyanin accumulation in *R*sc embryos isolated at successive developmental stages and cultured for 8 d in GM or in GM plus 10 μM ABA. B, Anthocyanin accumulation in stage 3 *R*sc embryos cultured for 8 d in GM alone, in GM supplemented with ABA, paclobutrazol (Pac), or 100 μM paclobutrazol with two concentrations of GA_3. C, Stage 3 embryos carrying other *R* alleles were cultured for 8 d on GM or GM supplemented with 10 μM ABA, 100 μM ancymidol (Ancy), or 100 μM paclobutrazol. Anthocyanin extraction and measurement are described in "Materials and Methods."

expressed in maturing embryos. The maturation mRNAs measured were the ABA-responsive genes *Glb*1, *Mem*, *Mec*, *Mlg*3, and *Dhn* (see "Methods and Materials"). These mRNAs have been shown to be in very low abundance in pre-maturation-stage embryos and to become highly abundant by embryo maturity. Each can be induced to accumulate precociously in cultured embryos given exogenous ABA treatment (Kriz et al., 1990; Thomann et al., 1992; White and Rivin, 1995a, 1995b). Because the VP1 transcription factor also participates in the control of these genes, the *Vp*1 mRNA level was also measured in each experiment.

The effects of GA synthesis inhibition on the message levels were compared with the effects of exogenous ABA treatment of cultured pre-maturation stage (19 DAP) wild-type embryos. With the exception of *Vp*1, none of the mRNAs was detectable in total RNA northern blots from freshly dissected 19 DAP embryos. To look for the induction of mRNA accumulation, samples of 19 DAP embryos were cultured for 3 d in either a hormone- and inhibitor-free medium (GM) or in medium supplemented with paclobutrazol alone, paclobutrazol plus GA_3, ABA alone, or ABA plus GA_3. In all media, the embryos remained ungerminated over the course of the treatment. Measurements of steady-state embryo message levels were made by northern blotting using total RNA samples.

The composite northern blot in Figure 7 shows that the inhibition of GA synthesis mimics the affects of ABA on the accumulation of maturation phase messages. RNA samples from the paclobutrazol-treated embryos showed considerable accumulation of all of the maturation phase mRNAs, while no accumulation was observed in the embryos cultured on minimal medium alone. The relative abundance

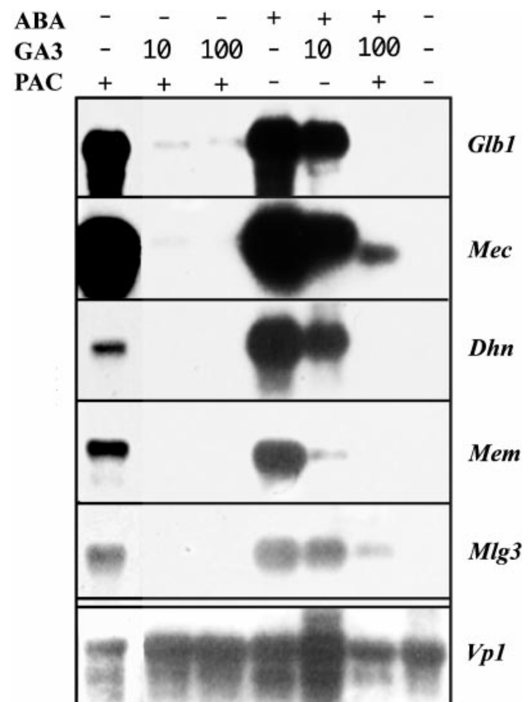

Figure 7. GA biosynthesis inhibition induces the accumulation of maturation mRNAs in cultured embryos. Pre-maturation wild-type embryos (19 DAP) were cultured for 3 d in GM supplemented with paclobutrazol (PAC) ± GA or ABA ± GA. Northern blots of RNA samples from each culture condition were probed with clones for the genes *Glb*1, *Mec*, *Dhn*, *Mem*, *Mlg*3, and *Vp*1. Lane 1, GM plus 100 μM paclobutrazol; lane 2, GM plus 100 μM paclobutrazol and 10 μM GA_3; lane 3, GM plus 100 μM paclobutrazol and 100 μM GA_3; lane 4, GM plus 10 μM ABA; lane 5, GM plus 10 μM ABA and 10 μM GA_3; lane 6, GM plus 10 μM ABA and 100 μM GA_3.

of the mRNAs and their total quantity was similar to the RNA profile of embryos treated with ABA. The mRNAs for *Glb*1, *Mec*, and *Mlg*3 were found at steady-state levels comparable to cultures that had been treated with 10 μM ABA, while the *Dhn* and *Mem* mRNAs accumulated to somewhat lower levels than with ABA treatment. The addition of 10 μM active GA obviated the effects of paclobutrazol treatment, severely reducing the steady-state levels of the maturation phase mRNAs.

The effects of exogenous GA on ABA-treated cultures were somewhat different: the addition of 10 μM GA_3 only moderately diminished the levels of *Glb*1, *Mec*, *Dhn*, and *Mlg*3 mRNAs, while the level of *Mem* mRNA was substantially reduced. When the embryos cultured with ABA plus 100 μM GA_3, the *Mec* and *Mlg*3 message levels were lower but still prominent, while the other ABA-inducible mRNAs were undetectable.

In all of the culture treatments, no consistent differences were observed in the steady-state level of *Vp*1 mRNA.

DISCUSSION

GA and the Suppression of Precocious Germination

The germination data from wild-type embryos demonstrated that GAs are required for the germination of immature embryos, and that the control of precocious germination involves marked changes in both endogenous GA levels and in the capacity for de novo GA synthesis as embryogenesis and maturation progress. In the early embryo, active GAs provide a positive signal for germination that must be counteracted by an ABA-responsive process. From our previous measurements of seed hormones, we know that stage 2 seeds contain active GAs and very little ABA (White et al., 2000). The rapid germination of these embryos in hormone-free medium and the failure of GA synthesis inhibitors to suppress their germination is probably due to the presence of the active GAs at the time of dissection. This contention is further supported by our previous observations that paclobutrazol treatment of the ear suppresses vivipary in ABA-deficient seeds when it is supplied prior to stage 2 (White et al., 2000).

Quiescence control in later embryogenesis may involve both low seed GA levels and a change in the inducibility of new GA synthesis, as shown by the change in germination behavior between stages 3 and 4 in cultured wild-type embryos. In contrast to the earlier stage, stage 3 embryos lack appreciable levels of active endogenous GAs and have a relatively high level of ABA (White et al., 2000). The suppression of germination caused by GA synthesis inhibitors indicates that de novo GA synthesis can and must take place in these cultured embryos to overcome the ABA signaling that maintains quiescence. It has been hypothesized that limiting water availability may be the most significant factor in maintaining quiescence at later stages of seed development, when ABA levels are very low. However, stage 4 embryos, which have relatively little ABA, still germinated at an extremely low rate in hormone-free culture medium. Seeds at this stage also have little active GA (White et al., 2000). Our results suggest that changes in the ability of older embryos to accumulate active GAs may be a factor in the maintenance of dormancy after ABA levels decline. The low level of stage 4 germination was suppressed by paclobutrazol, and full, rapid germination of the embryos was achieved with exogenous GA treatment. Thus, stage 4 embryos also appear to require GA for germination and to be GA sensitive. However, unlike younger embryos, they lack the capacity to accumulate a sufficient GA level for germination in culture. Changes in both synthesis and catabolic rates may contribute to this difference in germination behavior.

The contrast in the germination behavior of isolated wild-type and *vp*1-McW embryos is intriguing, indicating that the mutant, although quiescent on the ear, is in a different physiological state than the quiescent wild-type embryo. The rapid germination observed in stage 4 *vp*1 embryos is reminiscent of wild-type embryos at stage 2. It suggests that the mutant stage 4 embryos have GA levels sufficient to drive germination, either because they have higher active GA levels than wild-type stage 4 embryos, or because the ABA insensitivity of these mutants reduces the threshold level of GA required for germination. Such an inverse effect of ABA sensitivity and GA sensitivity has been noted in the germination of mature seeds of Arabidopsis, in which germination of the GA auxotroph *ga*1 is restored by either ABA auxotrophy or ABA-insensitive mutants (for review, see Koornneef and Karssen, 1994). Moreover, the ABA responsiveness of the ABA-insensitive ABI-1 mutant is increased in combination with the GA-insensitive *sly*1 mutation (Steber et al., 1998).

Also, unlike wild-type stage 4 embryos, the excised *vp*1 mutant embryos synthesized GA in culture, as seen by the effect of paclobutrazol treatment on germination rate. The apparent loss of GA accumulation capacity in maturing wild-type embryos and its retention in stage 4 *vp*1 mutants suggests that the decline of GA accumulation capacity during maturation may itself be an ABA-VP1 controlled response. This would be compromised in both types of *vp*1 mutants, while other quiescence factors are present in *vp*1-McW seeds. Analysis of gene expression in developing seeds and in transient assays has shown that the truncated *vp*1-McW protein is capable of activating a subset of the genes regulated by the wild-type VP1, and that it also retains the ability to repress germination gene expression in the aleurone (Carson et al., 1997).

GA and the Modulation of Maturation-Phase Gene Expression

Modulating the embryo GA level by paclobutrazol treatment or by exogenous GA addition had pronounced effects on the accumulation of mRNAs for a suite of maturation genes previously shown to be under transcriptional control by ABA and VP1. When treated with paclobutrazol, the steady-state accumulation of these mRNAs was very similar to the mRNA profile induced by exogenous ABA treatment. When active GA was added to paclobutrazol-treated cultures, these mRNAs did not accumulate. The effects of exogenous GA or GA synthesis inhibition on developmen-

tally specific anthocyanin accumulation also suggest that modulation of the GA/ABA balance alters the expression of the *C*1 gene, although the low level of *C*1 expression precluded direct examination of *C*1 mRNA levels in cultured embryos. The level of *Vp*1 mRNA was not altered by GA addition or synthesis inhibition or by exogenous ABA. These data suggest that the embryo GA acts, at some level, in opposition to the transcriptional stimulation of genes by ABA. However, the level at which gene expression is affected by GA modulation remains to be determined.

The opposing effects of GA and ABA on gene transcription have been well documented for the regulation of GA-induced germination genes in wheat and barley aleurone, particularly in the expression of hydrolytic enzymes such as α-amylase. In maize, VP1 has been shown to be a negative regulator of α-amylase expression in aleurone, as well as a positive gene regulator of maturation genes in the embryo Hoecker et al., 1995). To date, however, GA-responsive negative regulatory elements have not been demonstrated for any of the ABA-inducible promoters. The transcriptional motifs associated with ABA- and VP1-regulated genes are quite diverse (Shen et al., 1996), yet the responses of the various genes to paclobutrazol treatment were quite uniform. Therefore, we think it most likely that the inhibition of GA synthesis influences mRNA accumulation because it enhances ABA sensitivity, so there is a greater response from a limiting level of ABA, rather than from any direct effect of GA on transcription.

Evidence is accumulating for antagonism between ABA and GA signaling pathways in mature seeds. At the genetic level, the selection for second site suppressors of ABA-deficient and ABA-insensitive mutants of Arabidopsis has led to the recovery of mutations in the synthesis and signal transduction of GAs (Steber et al., 1998). Conversely, the selection for revertants of GA-sensitive non-germinating mutants led to the recovery of ABA mutants (Koornneef and Karssen, 1994). These results provide clear evidence that GAs are required for overcoming ABA-imposed dormancy and permitting germination in mature embryos. Furthermore, because the loss of a positive GA signaling component compensates for a loss of ABA sensitivity, they support the notion that positive GA signaling antagonizes ABA signal pathways in mature seeds.

Antagonism of GA and ABA signaling also appear to be involved in the regulation of GA-induced gene expression. During the germination of cereals, GA secreted by the embryo induces the transcription of genes for α-amylase and other hydrolytic enzymes, and the premature transcription of these genes is suppressed by the presence of ABA (for review, see Jacobsen et al., 1995). The ABA-regulated protein kinase PKABA causes a deep reduction in the expression of GA-inducible germination genes when it is overexpressed in barley aleurone cells, indicating that this signaling component is one means through which ABA can suppress GA-inducible genes (Gomez-Cadenas et al., 1999). Expression from the GA-inducible α-amylase promoter was also suppressed by the overexpression of HvSPY, a putative negative regulator of GA action in barley aleurone. Interestingly, in this experiment, expression from the ABA-inducible dehydrin promoter was seen to increase (Robertson et al., 1998). HvSPY is the barley equivalent of the Arabidopsis SPINDLY gene, whose mutant alleles cause GA hypersensitivity (Jacobsen and Olszewski, 1996), so these results suggest that reducing GA signaling by overexpressing a negative regulator can have a positive effect on an ABA-inducible pathway.

Our results with maize embryos, both in culture and in vivo, shows that GA is synthesized and there is a change in active GA accumulation during the development of the immature embryo that may reflect alterations in the synthesis rate, in catabolism, or in both. The alteration in active GA levels forms a counterpoint to the change in ABA level. The capacity of the developing maize embryo for germination and for the expression of ABA-regulated genes is temporally determined by this changing balance of hormones. The simplest hypothesis to explain the general antagonism of GA to ABA responses is that GA signaling antagonizes some common, early point in the ABA signaling pathway during embryogenesis. Thus, when ABA levels are low, active GA signaling can block ABA responses, while a reduction in the GA level appears to lower the threshold for ABA effectiveness. This basic model is consistent with the above-cited studies examining mature embryos.

One inconsistency is the results obtained when exogenous GA was added to cultures that were also exposed to high levels of exogenous ABA. The reduction in maturation mRNA levels were much weaker than those observed when GA was added to paclobutrazol-treated cultures. Also, the level of change in steady-state accumulation was not uniform for the different genes, as it was when GA was added to paclobutrazol-treated embryos. To account for this weakened and gene-specific response, we must propose that GA signaling is less effective in overcoming ABA signaling when ABA concentrations are high, and that it may also influence individual gene expression at later steps. We are currently testing these ideas by examining which aspects of gene expression in immature embryos are affected by alterations in GA/ABA levels.

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