Plant Physiol. (1990) 94, 1172–1179
0032-0889/90/94/1172/08/$01.00/0

Received for publication May 8, 1990
Accepted July 3, 1990

Three Classes of Abscisic Acid (ABA)-Insensitive Mutations of *Arabidopsis* Define Genes that Control Overlapping Subsets of ABA Responses[1]

Ruth R. Finkelstein* and Chris R. Somerville

Department of Biological Sciences, University of California at Santa Barbara, Santa Barbara, California 93106 (R.R.F.), and Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Wild type and three abscisic acid (ABA)-insensitive mutants of *Arabidopsis* (*ABI1*, *ABI2*, and *ABI3*) were compared for their ability to respond to ABA for a variety of ABA-inducible responses throughout the life cycle of the plants. The responses tested included effects on seedling growth, proline accumulation in seedlings, ABA-regulated protein synthesis in plantlets, and seed storage protein and lipid synthesis and accumulation. The *abi1* and *abi2* mutants showed reduced sensitivity to ABA for inhibition of seedling growth, induction of proline accumulation, and alterations in protein synthesis patterns during vegetative growth, but had wild type levels of storage reserves. In contrast, the *abi3* mutant had wild type sensitivity for induction of proline accumulation and was only slightly less responsive to ABA with respect to effects on seedling growth and changes in patterns of protein synthesis. The major effects of this mutation were on seed development. Seeds of the *abi3* mutant had two-thirds of the wild type level of storage protein and one-third the wild type level of eicosenoic acid, the major fatty acid component of storage lipids in wild type seeds. These results show that none of the *abi* mutants is insensitive for all ABA-inducible responses and that the *abi3* effects are not seed-specific. Comparison of the degree of ABA sensitivity of monogenic mutant lines with that of digenic mutant lines carrying pairwise combinations of the *abi* mutations suggests that ABA responses in mature seeds are controlled by at least two parallel pathways.

The plant growth regulator ABA affects many aspects of plant growth and development, including embryo development, seed dormancy, water relations, and tolerance of a variety of environmental stresses (reviewed in ref. 23). Based on physiological and biochemical criteria, these responses have been subdivided into two classes: rapid (<5 min) and slow (>30 min) responses. The rapid responses, such as stomatal closure, involve changes in K^+ ion flux and can discriminate between the enantiomers of ABA, responding only to S-ABA, the naturally occurring form (22). Many of the slow responses, such as inhibition of seed germination and induction of seed reserve accumulation, appear to involve changes in gene expression and respond equally to S- and R-ABA, but the mechanism and site of ABA action is still obscure (23). Studies of the effects of ABA and the fungal toxin fusicoccin on a number of plant processes (*e.g.* germination, stomatal closure, and cell elongation) have shown that in all cases the antagonistic effects of these growth regulators are reflected in opposite effects on ion flux (reviewed in ref. 18). Thus the slow responses may represent an amalgam of ABA effects acting at many different levels, ranging from rapid effects on ion flux to slower effects on gene expression.

A number of mutants of *Arabidopsis* with altered sensitivity to ABA were previously isolated by selecting for germination in the presence of exogenous ABA (17). These mutants were placed in three complementation groups, designated *abi1*, *abi2*, and *abi3*. The initial characterization showed that mutations at all three loci resulted in decreased seed dormancy and up to a 10-fold reduction in sensitivity to exogenous ABA for inhibition of germination. None of the mutants showed a complete loss of response to ABA, indicating that they were either leaky mutations or affected only one of several ABA response pathways. The *abi1* and *abi2* mutants differed from *abi3* mutants in that they, like a previously isolated ABA-deficient mutant (16), were prone to wilting (17). Thus, single mutations in either *ABI1* or *ABI2* could disrupt both rapid and slow ABA responses, but *ABI3* was required for only a subset of those responses requiring *ABI1* and *ABI2* action. Because these mutations provide a basis for identifying classes of ABA response that overlap, but differ from, the slow *versus* rapid response classification scheme, a goal of the present studies was to devise a scheme of ABA action consistent with the phenotypes of these mutants. We have extended the physiological characterization of these mutants to include more responses throughout the life cycle in an effort to further define common characteristics of the subset of responses affected by each *abi* mutation as well as the timing of *ABI* gene action. In addition, we have compared ABA sensitivity of double mutants to determine whether these loci are acting in the same or overlapping pathways.

MATERIALS AND METHODS

Plants

Arabidopsis thaliana seed stocks carrying the following mutant alleles were a generous gift of M. Koornneef: *abi1*

[1] This work was supported in part by a grant from the United States Department of Energy (AC02-76ER01338). R.R.F. was supported in part by a National Science Foundation postdoctoral fellowship (DCB-8608512).

(isolation number AII), *abi2* (EII), *abi3* (CIV), and *aba-1* (A26). All are ethyl methanesulfonate-induced mutants derived from the Landsberg "erecta" line (16, 17). Plants were grown to maturity under continuous fluorescent illumination (100–150 μmol m^{-2} s^{-1}) at 22°C on a mixture of vermiculite, perlite, and sphagnum (1:1:1) irrigated with mineral nutrients (8). For germination or seedling growth assays, seeds were surface sterilized in 5% hypochlorite, 0.02% Triton X-100, then rinsed four to five times with sterile water before plating on minimal medium with or without ABA (mixed isomers, Sigma) at the indicated concentrations. Minimal medium was composed of mineral nutrients (8) and 0.7% agar. Petri dishes were incubated at 24°C in continuous light (30 μmol m^{-2} s^{-1}). All seeds were incubated at least 2 d at 4°C after sowing before transfer to growth chambers.

Proline Assays

Seedlings were grown 9 d on minimal plates (until the first two true leaves expanded), then transferred to control medium (50 mM sucrose, 1 mM glutamate) with 0, 5, 25, or 50 μM ABA for 24 h. The sucrose and glutamate were included to ensure an adequate supply of precursors for proline accumulation. Entire seedlings were weighed, frozen in liquid nitrogen, and stored at −80°C until extraction. Proline content was determined essentially as described by Bates *et al.* (3). Plants were homogenized in 3% sulfosalicylic acid (50 μL/mg FW[2]) and extracts were clarified by centrifugation for 10 min at 13,600 *g*. Extracts were mixed with glacial HOAc and acidic ninhydrin reagent (1:1:1) and incubated at 100°C for 1 h. The reaction was chilled in an ice bath, then extracted with an equal volume of toluene and the absorbance of the toluene phase measured at 520 nm.

Protein Synthesis in Seedlings

In vivo synthesized protein was labeled with a 12 h pulse of ^{35}S-methionine (5 μCi/plant) applied to the adaxial surface of leaves of 2-week-old plants in 10 μL of 0.025% Triton X-100 ± ABA. Plants were rinsed at harvest to remove excess label, then frozen in liquid nitrogen and stored at −80°C until extraction. Protein was extracted essentially as described by Hurkman and Tanaka (10). Tissue was ground in 0.7 M sucrose, 0.5 M Tris-HCl (pH 9.4), 50 mM EDTA, 0.1 M KCl, 2% 2-mercaptoethanol, 2 mM PMSF (grinding volume ranged from 4–8 mL per g FW). To identify heat-stable proteins, a portion of each homogenate was boiled for 10 min, then placed on ice. All extracts were clarified by centrifugation for 8 min at 13,600 *g*. Supernatants were extracted with an equal volume of phenol and proteins were precipitated from the phenol phase with 5 volumes of 0.1 M NH_4OAc in methanol at −20°C overnight. The protein pellet was washed with acetone, dried, and resuspended in SDS-PAGE sample buffer. Incorporation into protein was assayed by determining the percentage of total radioactivity that was TCA-precipitable. Equal cpm of extracts were separated on SDS-PAGE (13) and gels were equilibrated with En^{3}Hance (DuPont), dried, and fluorographed.

[2] Abbreviation: FW, fresh weight.

Embryo Culture

Five- to 6-d-old siliquae (containing early cotyledon stage embryos) were harvested for embryo culture. A dissecting needle was used to scoop seeds out of siliquae into a pool of sterile water on a microscope slide. At this stage much of the seed volume was liquid endosperm and gentle pressure with a cover slip expelled the embryos from the seeds. Embryos were collected on 45-μm Nitex nylon mesh and transferred to MS salts (19) supplemented with 2% sucrose, 0.7% agar, and with or without 10 μM ABA. After 24-h culture at 24°C in continuous light (30 μmol m^{-2} s^{-1}), embryos were rinsed off the Nitex mesh, collected in 1.5-mL microfuge tubes, and frozen in liquid nitrogen.

Storage Protein mRNA Quantitation

RNA was isolated, and the abundance of specific transcripts was quantitated by dot-blot assays essentially as described previously (5). The insert DNA from the cruciferin cDNA probe pC1 (21) was labeled with [α-^{32}P]deoxycytidine 5′-triphosphate (3000 Ci $mmol^{-1}$) by random priming (9) to a specific activity of 10^8 cpm μg^{-1}, and the rDNA probe pRE12 (4) was labeled with [α-^{32}P]deoxycytidine 5′-triphosphate by nick translation to 10^7 cpm μg^{-1}. The cruciferin cDNA was also used to probe Northern blots of total embryo RNA to be certain that only one size class of mRNA was being detected in this heterologous system.

Extraction of Seed Proteins

Total soluble seed protein was extracted from approximately 5 mg of mature seeds of each genotype in 50 μL mg^{-1} FW of 63 mM Tris-HCl (pH7.8), 10 mM 2-mercaptoethanol, 20% glycerol. Proteins were separated by SDS-PAGE (13), and relative band intensities were quantitated by densitometric scanning of a photographic negative of the Coomassie blue-stained gel.

Fatty Acid Analysis

Fatty acid composition was determined for mature seeds of all genotypes and for wild type and *abi3* seeds at a series of developmental stages. Immature seeds were harvested as described for embryo culture. Fatty acids of seed lipids were extracted and quantitated as described previously (7).

RESULTS

Seedling Growth

The initial characterization of the *abi* mutants indicated that mutations at all 3 *ABI* loci reduced seed dormancy and sensitivity to exogenous ABA with respect to inhibition of germination (17). In those experiments, the effects of the *abi* mutations on ABA-sensitivity of seedling growth were assessed by measuring the FW of seedlings 12 d after sowing on various concentrations of ABA. To eliminate the effects of ABA on germination from this assay, we germinated seed on hormone-free medium, then transferred seedlings to either fresh minimal medium or medium supplemented with 10 μM ABA. Seedlings were transferred after 2 d (when radicles were

first visible), or after 3 to 4 d to determine whether ABA sensitivity changed during the early stages of seedling growth. All seedlings were harvested 12 d after sowing. Figure 1 shows the FW of seedlings grown on ABA as a percentage of that on minimal medium. Growth of the wild type seedlings was inhibited regardless of when they were transferred to ABA. In contrast, the *abi1* and *abi2* mutants showed little or no inhibition of growth (Fig. 1). Indeed, growth of the *abi1* mutant was actually stimulated by ABA. The *abi3* mutant displayed a level of sensitivity between that of the other *abi* mutants and the wild type seedlings, indicating that the effects of this mutation do indeed extend past germination.

Proline Accumulation

To assay the ABA-sensitivity of the mutants at a later stage in vegetative growth, their ability to accumulate proline in response to exogenous ABA was tested. Many plants accumulate proline in response to a variety of stresses and some species will accumulate proline in the absence of stress when treated with ABA (2). Wild type *Arabidopsis* plants showed a 10-fold increase in proline levels after 24-h culture on 50 μM ABA (Fig. 2). *Abi3* plants showed a comparable increase in proline under these conditions. In contrast, proline levels in the *abi1* and *abi2* mutants increased only four- and six-fold, respectively. Although there was not a statistically significant difference between the amount of proline that accumulated in any line at 25 and 50 μM ABA, the mean was higher at 50 μM, suggesting that the responses saturate at similar ABA levels in all genotypes, but the magnitude of the response is reduced.

Protein Synthesis in Vegetative Tissues

To determine whether the *abi* mutations affected a large number of ABA-regulated responses, the effects of ABA on total protein synthesis in leaves of wild type and mutant plants were examined. Two-week-old plants were transferred to medium containing 50 μM ABA for 2 d, then given a 12-h pulse of ^{35}S-methionine before harvest and extraction of total proteins. ABA treatment reduced overall incorporation into protein by about 25% in wild type plants, but only approximately 10% in the mutants (data not shown). Because some ABA-induced proteins have been shown to be resistant to denaturation by treatment at 100°C in various cereals (11), the composition of protein extracts before and after removal of proteins denatured by boiling were compared by SDS-PAGE. Differences between the spectrum of proteins present in control and ABA-treated plants were most readily apparent in the heat-treated samples (Fig. 3). This experiment showed numerous ABA-induced or -repressed proteins whose ABA regulation was disrupted in the *abi1* and *abi2* mutants. For instance, in Figure 3, band a is ABA-induced in wild type but not in any *abi* mutants, whereas bands b, c, f, and h are decreased in wild type and *abi3* plants, but not in *abi1* and *abi2* mutants treated with ABA. In contrast to the *abi1* and *abi2* mutants, *abi3* mutants showed altered regulation of only a subset of ABA-regulated proteins (*e.g.* Fig. 3, bands a and g). Furthermore, none of the mutations resulted in complete loss of ABA regulation (*e.g.* in Fig. 3, bands d and e are still ABA-responsive in all three *abi* mutants). These results support the previous observations that the *abi1* and *abi2* mutations affect many processes in vegetative growth, but indicate that they may not be required for all ABA-inducible phenomena in vegetative tissue. In addition, these results provide evidence that *abi3* is also expressed in vegetative tissue but apparently affects only a subset of those responses regulated by *abi1* and *abi2*.

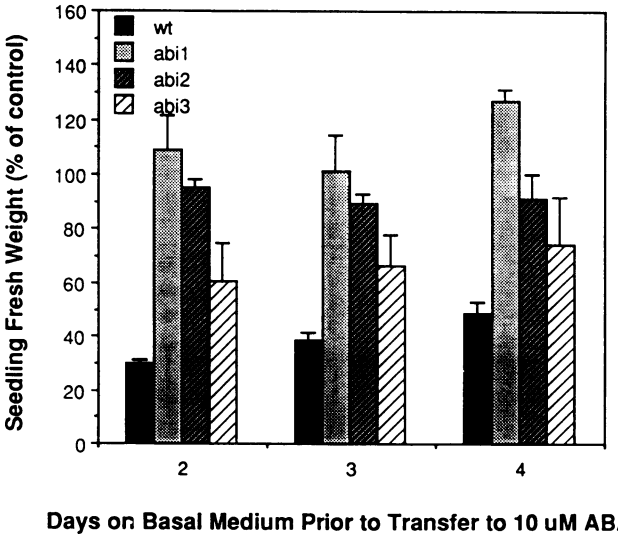

Figure 1. ABA sensitivity of seedling growth. Fresh weight of seedlings grown on 10 μM ABA is expressed as a percentage of those grown on hormone-free medium. Values shown are mean ± SE of duplicate samples composed of 12 to 30 seedlings each.

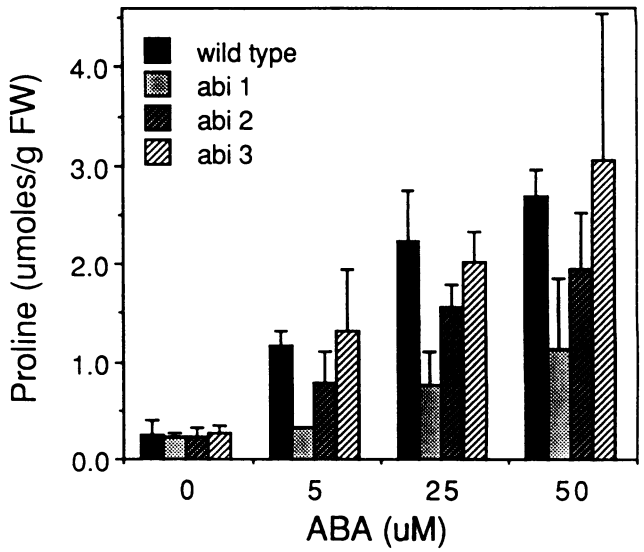

Figure 2. ABA dose response of proline accumulation. Plantlets were cultured on 0, 5, 25, or 50 μM ABA for 24 h before harvest and extraction. Data shown are mean ± SE of duplicate samples in three independent experiments.

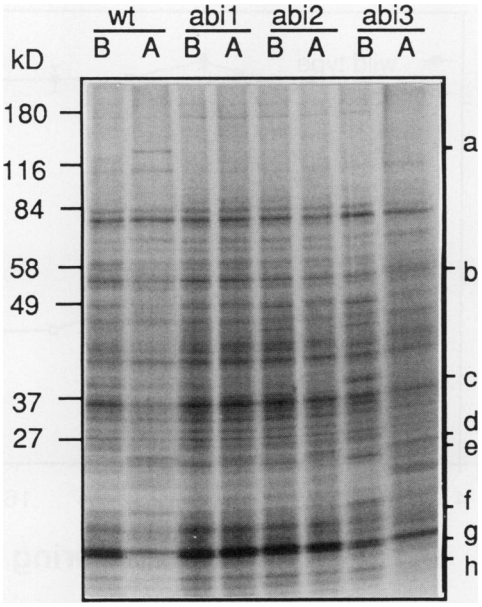

Figure 3. ABA-regulated protein synthesis in seedlings. Following 2 d culture ±50 μM ABA, seedlings were pulse-labeled with ^{35}S-methionine for 12 h. Heat stable proteins were separated by SDS-PAGE and fluorographed. B, extracts from seedlings grown on hormone-free medium; A, extracts from ABA-treated seedlings. Bands a and d are ABA induced; b, c, e, f, g, and h are ABA repressed.

Seed Storage Reserve Synthesis

ABA has been implicated in regulating accumulation of seed storage reserves in a variety of angiosperms. To determine whether the *abi* mutations altered this aspect of seed metabolism, the effects of these mutations on accumulation of storage proteins and storage lipids was examined.

The major storage proteins of *A. thaliana* are encoded by a family of four genes sharing 75% sequence homology (20). The deduced coding sequence of one of these genes is 86% homologous to the coding sequence of a cDNA for one of the cruciferin genes of *Brassica napus*. In *B. napus,* ABA stimulates expression of these genes in isolated embryos, and the timing of cruciferin mRNA and ABA accumulation show similar patterns *in situ* (5). Therefore, we compared the ABA-responsiveness of storage protein mRNA accumulation in wild type and mutant seeds. Developing seeds of the wild type and the *abi1* and *abi2* mutants had comparable levels of cruciferin mRNA both *in situ* and in response to exogenous ABA, but developing seeds of the *abi3* mutant had reduced levels of this mRNA family in both conditions (Fig. 4). A previous comparison of the developmental timing of expression of the cruciferin-homologous genes showed a slight delay (*i.e.* 2 d) of transcript accumulation in seeds of the *abi3* mutant (20), which could account for the difference in mRNA levels between wild type and the *abi3* mutant at the start of the experiment (*i.e.* 0 d). However, even peak transcript levels are lower in *abi3* seeds than in the other genotypes (data not shown); this is reflected in a slight reduction of storage protein accumulation at seed maturity. Densitometric analysis of Coomassie blue-stained gels (Fig. 5) showed that the cruciferin proteins comprised 30 to 35% of total seed protein in seeds of the *abi3* mutant, but 50 to 55% of seed protein in the other genotypes. It is notable that the *aba* mutant has wild type levels of these proteins even though its endogenous ABA levels are only 5% of those in wild type seeds (12). This indicates that although ABA can regulate this gene (as indicated by the results of *in vitro* culture experiments), the high endogenous ABA levels present during development of wild type seeds are not absolutely required for its expression. A similar conclusion was reached regarding regulation of the *Brassica* genes, using embryo culture rather than mutations to manipulate ABA levels (6).

The other major storage reserve of *Arabidopsis* is triacylglycerol. As in other members of the Brassicaceae, the triacylglycerol fraction in *Arabidopsis* seeds is highly enriched in fatty acids with 20 or more carbon units (1). We have previously demonstrated ABA-inducibility of long-chain fatty acid accumulation in *B. napus* (7). Therefore, we assayed the effect of the various *abi* mutations on storage lipid synthesis by measuring the accumulation of long chain fatty acids. Comparison of the various *Arabidopsis* genotypes shows that eicosenoic acid (20:1, the major storage form) is threefold lower in *abi3* than in the other genotypes (Table I). Consequently, 18:1 and 18:2 (the precursor and alternate products, respectively) constitute a larger percentage of seed fatty acids in the *abi3* mutant. Quantitative analysis of seed fatty acid content (μg per seed) shows no statistically significant differences between wild type and any of the *abi* mutants, indicating that the *abi3* mutation preferentially affects the fatty acid elongation pathway. Comparison of the developmental time course

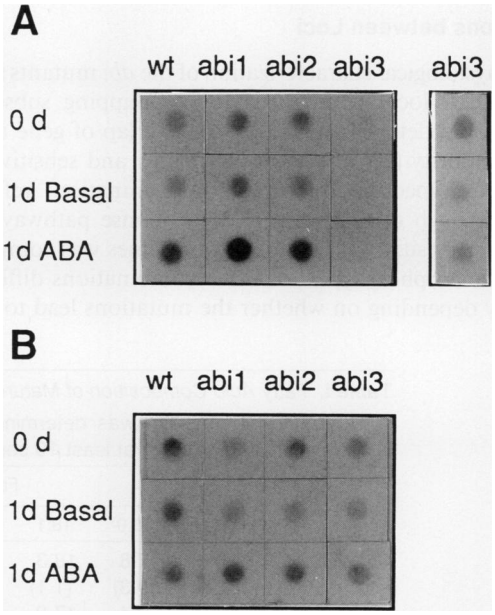

Figure 4. ABA-induction of cruciferin mRNA accumulation in 5 DAF (early cotyledon stage) embryos. Embryos were cultured 24 h ± 10 μM ABA before harvest and RNA extraction. A, cruciferin mRNA accumulation in wild type and *abi* mutants was compared on dot blots (2 μg total RNA/dot) probed with a *B. napus* cDNA clone. Right panel, fivefold longer exposure of the *abi3* dots. B, accuracy of RNA loading was tested by hybridization to an rDNA probe.

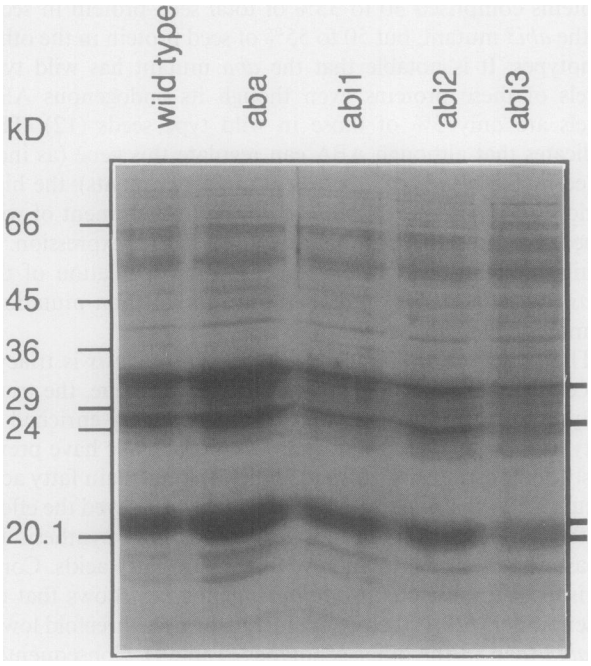

Figure 5. SDS-PAGE of total soluble protein in mature seeds. Heavy bars, storage protein subunits.

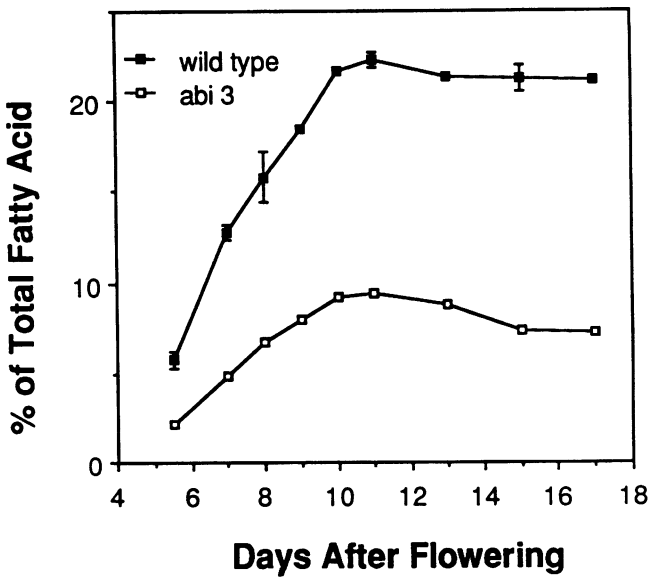

Figure 6. Accumulation of eicosenoic acid (20:1) during seed development. Values shown are mean ± SE of two to four samples per timepoint.

of eicosenoic acid accumulation shows that the *abi3* mutant has lower levels than wild type as early as 5 DAF, indicating that *abi3* gene action is normally required at least this early (Fig. 6).

Interactions between Loci

The physiological characterization of the *abi* mutants shows that the *ABI* loci are required for overlapping subsets of responses. To determine whether the overlap of gene action at seed maturity (*i.e.* effects on dormancy and sensitivity to ABA with respect to inhibition of germination) reflected activity through one or more ABA response pathways, the degree of insensitivity of double mutant lines was compared. The predicted phenotypes for these combinations differ significantly depending on whether the mutations lead to complete loss of activity for the affected gene product (*i.e.* are null mutations) or retain some residual activity. A double null mutant should show the same degree of insensitivity as that of the epistatic monogenic mutant if these genes are acting in the same pathway but should be much more insensitive than either single mutant if the genes are acting through different pathways. In contrast, if the mutations are leaky, the double mutant phenotype should be additive if the mutations affect steps in different pathways, but multiplicative (*i.e.* more severe) if they affect steps in the same pathway. Note that regardless of whether the mutations are null or leaky, the appearance of two phenotypic classes of double mutants is consistent with a model in which these genes define more than one pathway regulating the process.

Double mutant lines were constructed by crossing all pairwise combinations of monogenic *abi* lines, allowing the F1 plants to self-pollinate and screening the F2 plants for double

Table I. *Fatty Acid Composition of Mature Arabidopsis Seeds*

Fatty acid composition was determined by GC analysis of fatty acid methyl esters. Values are average of 10 replicates of at least 10 seeds each. SE are in parentheses below the means.

Genotype	Fatty acid (% of total)								Fatty acid/seed (μg)
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	24:1	
Wild type	6.3	2.8	18.3	28.1	20.2	22.3	0.9	1.1	6.2
	(0.3)	(0.3)	(1.1)	(0.5)	(0.5)	(1.1)	(0.2)	(0.3)	(0.7)
aba	7.2	2.4	17.0	32.8	16.0	22.0	1.1	1.9	5.5
	(0.7)	(0.2)	(4.0)	(1.9)	(0.5)	(1.2)	(0.3)	(1.8)	(1.5)
abi1	6.7	2.7	17.6	29.5	19.4	21.9	1.0	1.2	5.6
	(0.7)	(0.3)	(2.7)	(0.9)	(1.0)	(1.3)	(0.3)	(0.5)	(1.3)
abi2	6.0	2.5	16.9	28.4	20.6	23.2	1.0	1.2	6.1
	(0.2)	(0.1)	(2.4)	(0.5)	(1.4)	(1.4)	(0.2)	(0.5)	(0.7)
abi3	8.3	2.2	23.7	36.7	20.9	7.6	0.5	—	4.7
	(0.9)	(0.3)	(3.0)	(0.9)	(2.1)	(0.6)	(0.2)	—	(1.7)

mutants. The *ABI* loci are unlinked (ref. 14; unpublished observations) and thus should not present any intrinsic difficulties for construction of the double mutants. F2 individuals homozygous for either of the recessive mutations (*abi2* and *abi3*) or heterozygous for the dominant *abi1* mutation were selected for their ability to germinate in the presence of 10 μM ABA. Because all three mutations affect ABA inhibition of germination, determination of the genotype at each locus for these F2 individuals required backcrossing to appropriate tester lines. In addition, *abi3* homozygotes could be identified as those with a threefold decrease in seed eicosenoic acid content. Using these criteria, we identified *abi1 abi2* and *abi1 abi3* double homozygotes. However, we were unable to isolate any *abi2 abi3* double homozygotes from the original F2 population or even from the selfed progeny of *abi2/ + abi3/ abi3* lines, 25% of whose progeny should be the double homozygote if this genotype is viable.

A dose-response curve for ABA-inhibition of germination shows that while the *abi1 abi2* combination is slightly more insensitive than *abi1* or *abi2* alone, the *abi1 abi3* mutant is extremely insensitive to ABA (Fig. 7). However, the *abi1 abi3* mutant is still not completely insensitive to ABA since it germinates more slowly on increasing concentrations of ABA (data not shown). Although the *abi2 abi3* double mutant appears to be lethal (manuscript in preparation), the progeny of self-pollinated *abi2/ + abi3/abi3* plants (67% *abi2/ + abi3/abi3*, 33% *+/+ abi3/abi3*) also exhibit a massive decrease in sensitivity relative to either *abi2* or *abi3* mutant seeds. This indicates that although *abi2* alone is a recessive mutation with respect to ABA inhibition of germination, one wild type copy of this gene is no longer sufficient for full ABA response in an *abi3* background.

DISCUSSION

The *abi* mutants of *Arabidopsis* were selected for their ability to germinate in the presence of inhibitory concentrations of exogenous ABA (17). The initial characterization showed that all three classes of mutants (*abi1, abi2,* and *abi3*) also failed to become dormant (17), a process that previous studies had shown to require embryonic ABA midway through seed development (12). Recently, Koornneef *et al.* (15) have demonstrated that loss of dormancy in all three classes of *abi* mutants can first be detected midway through seed development, implying that all three loci are active during seed development. The mutants differed in their water relations; only *abi1* and *abi2* plants were prone to wilting, presumably as a result of impaired stomatal regulation. Thus, previous studies were consistent with a model in which *ABI3* was involved in seed-specific response, while *ABI1* and *ABI2* appeared to be required for all responses tested. In order to test this model, we have assayed a variety of ABA-inducible responses throughout the *Arabidopsis* life cycle to better define the timing of gene action for the *ABI* loci.

Effects on Seed Development

Comparison of the effects of the *abi* mutations on storage reserve accumulation indicates that, despite the fact that action of all three *ABI* loci during seed development is required for induction of dormancy, only the *abi3* mutant has a detectable effect on the quality or composition of storage protein or lipid. In view of the pronounced effect of the *abi3* mutation it is somewhat surprising that ABA deficiency alone is not sufficient to impair storage reserve accumulation. However, recent studies by Koornneef *et al.* (15) show that the *aba abi3* double mutant (combining deficiency with insensitivity) produces seed that fail to desiccate, lose viability, and produce almost no storage proteins. Furthermore, they demonstrated that maternal ABA is sufficient for acquisition of desiccation tolerance since *aba abi3* seeds produced by self-fertilization of *aba/+,abi3/abi3* plants desiccated and were fully viable. Application of ABA or an ABA analog to either roots or leaves of *aba abi3* plants also increased the desiccation tolerance of their progeny, but did not restore seed storage protein accumulation. The observation that dormancy requires embryonic ABA while induction of desiccation and desiccation tolerance require maternal ABA indicates that these processes are being controlled by events in different tissues, but does not address the nature of these control processes. It is noteworthy that although an effect on storage protein accumulation could be expected on the basis of the *abi3* mutant phenotype, the effects on desiccation and desic-

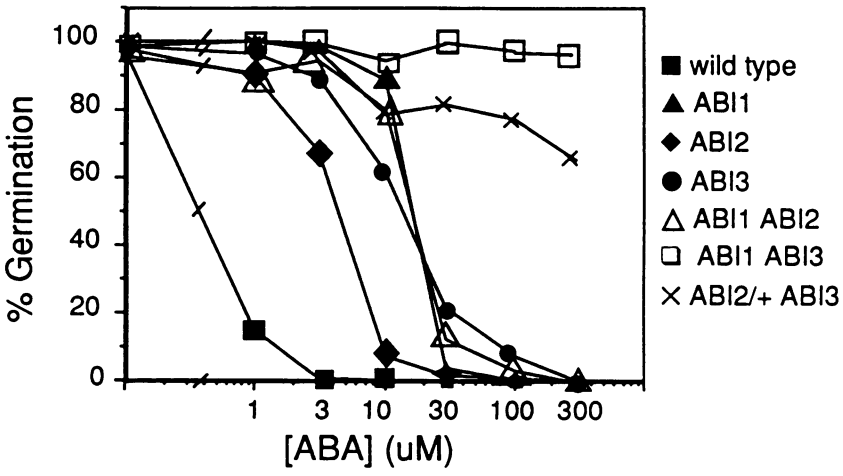

Figure 7. Effect of ABA on germination of mono- and digenic *abi* mutants. Germination percentage was scored 4 d after plating on minimal media containing 0, 1, 3, 10, 30, 100, or 300 μM ABA. Values shown are mean ± SE of four experiments, each with 30 to 150 seeds per treatment.

cation tolerance are cryptic in that they are not predicted by the phenotypes of either *aba* or *abi3* mutants.

One possible explanation suggested by Koornneef *et al.* (15) for the dramatic phenotype of the *aba abi3* seeds is that, in a wild type background, the reduction in endogenous ABA level caused by the *aba* mutation is above the threshold level required for normal seed development. According to this model, this threshold level is increased in the *abi3* mutant, and the decrease in ABA levels due to the *aba* mutation prevents the *aba abi3* double mutant from reaching this higher threshold requirement for ABA. Seeds of the monogenic *abi3* mutant normally have increased ABA levels and may be able to partially compensate for their insensitivity (*i.e.* increased threshold requirement) in this manner. However, not all combinations of ABA deficiency and insensitivity result in a severely mutant phenotype. For example, the phenotype of the double mutant *aba abi1* is no more severe than that of either monogenic mutant. Alternatively, it is possible that the mechanisms regulating seed development have a substantial level of redundancy. *ABI3* may act in a regulatory pathway that responds to a variety of physiological cues in seed development, including ABA, while perception of ABA may trigger multiple transduction pathways, one of which requires *ABI3* action. A deficiency of either ABA or the *ABI3* gene product could be compensated by action of an alternate signal or pathway, but loss of both would result in a severely mutant phenotype.

Effects on Vegetative Tissues

We tested the effects of the *abi* mutations on three ABA-regulated responses occurring during vegetative growth: (a) inhibition of seedling growth; (b) induction of proline accumulation; and (c) ABA-regulated changes in protein synthesis. Although each of these responses may be subject to multiple levels of control, we have focused on ABA-induction to assay the role of the *ABI* gene products. Effects of the *abi3* mutation were much more limited during vegetative growth than during seed development. ABA inhibition of seedling growth and some ABA-regulated effects on protein synthesis were slightly reduced in the *abi3* mutants as compared with the wild type, but proline accumulation was similar to that in wild type plants. In contrast, the *abi1* and *abi2* mutants were insensitive to ABA inhibition of seedling growth and induction of proline accumulation and have lost most, but not all, ABA-regulation of protein synthesis.

Considered as a whole, the studies reported here, along with earlier work by Koornneef *et al.* (17), show that *ABI3* is active throughout the life cycle, but appears to control more processes during seed development than during vegetative growth. *ABI1* and *ABI2* are required for relatively few processes during seed development, but control many ABA-regulated responses during vegetative growth (Fig. 8). None of these loci appears to be required for all ABA responses tested to date, nor is expression of any of them restricted to a single developmental stage. However, these experiments were conducted with only one representative allele at each locus, none of which were known to be null mutations, so it is possible that our results underestimate the requirements for these gene products.

Models for the Role of the *ABI* Genes

Any model to explain the role of the *ABI* genes must account for the phenotypes of digenic mutants combining either pairs of ABA response mutations or ABA response and ABA biosynthesis mutations, as well as the phenotypes of the monogenic mutant lines. Similar to the results described above for combinations of *abi* and *aba* mutations, pairwise combinations of the *abi* mutations produced two phenotypic classes. The *abi1 abi2* double mutant showed approximately the same degree of insensitivity for inhibition of germination as either *abi1* or *abi2* single mutants, whereas the *abi1 abi3* double mutant was much more insensitive than either the *abi1* or *abi3* single mutants. However, the *abi1 abi3* mutant differed from the *aba abi3* mutant in that it did not eliminate desiccation tolerance or storage reserve accumulation (data not shown). The *abi2 abi3* double mutant appeared to be lethal, but even *abi2/+abi3/abi3* seeds showed a massive reduction in ABA sensitivity of germination inhibition.

The results of these double mutant studies could be ex-

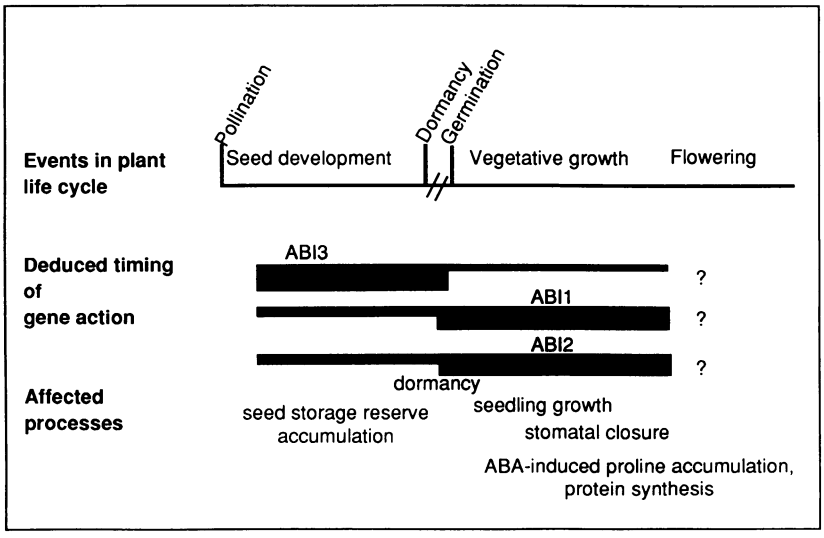

Figure 8. Summary of physiological characterization of *abi* mutations. Width of bars reflects number of processes affected by mutation.

plained most easily by either a decrease below a threshold level in the amount of a required precursor in a single pathway or reduced activity of two parallel signal transduction pathways. In the first case, the response pathway could be composed of a cascade of amplifying steps leading toward production of a molecule required for response. A leaky mutation at any of these steps could still allow sufficient amplification to reach a required threshold of this molecule. Combination of mutations affecting later steps in the cascade would not reduce the level of the active molecule below the threshold, but combination of mutations affecting either two early steps or early and late steps would. Assuming that *ABI3* is required for an earlier step than *ABI1* or *ABI2,* this model could explain the relative ABA sensitivity with respect to germination of the monogenic *abi* lines and the digenic combinations of *abi1 abi3* and *abi1 abi2*. However, it does not account for the observation that *abi1* affects a subset of responses not altered in *abi3* and vice versa. In the second model, the responses are controlled by parallel regulatory pathways affecting overlapping subsets of responses. As discussed earlier, it is not possible to assign the *ABI* gene products to pathways solely on the basis of the double mutant phenotypes without knowing whether these are null or leaky mutations. However, two other lines of evidence suggest that *ABI1* and *ABI3* are required for different pathways controlling ABA response. First, these mutations affect overlapping but different subsets of responses. Second, the *aba abi1* phenotype is much less severe than the *aba abi3* phenotype, indicating that the reduction in ABA level due to the *aba* mutation does not reveal any cryptic overlap between responses requiring *ABI1* and *ABI3* action. While the available experimental evidence does not allow us to discriminate between the models outlined above, they are not mutually exclusive. The combinatorial control afforded by the latter model is particularly attractive because it would be well-suited to permit fine-tuning of the responses under the noncontrolled conditions encountered by wild type plants growing outside the laboratory.

The results described in this article and previously (15, 17) allow us to make several predictions about when and where these gene products are active. Their activity may be regulated by presence of the *ABI* gene products themselves or by interaction with other products whose availability is developmentally regulated. Molecular probes for the *ABI* gene products would allow us to distinguish between these possibilities and might provide further clues about their function.

ACKNOWLEDGMENTS

We thank M. Koornneef for providing seeds of the *abi* and *aba* mutant lines, M. Delseny for the rDNA done, and M. Crouch for the cruciferin cDNA clone.

LITERATURE CITED

1. **Appelqvist LA** (1975) Biochemistry and structural aspects of storage and membrane lipids in developing oilseeds. *In* T Galliard and EI Mercer, eds, Recent Advances in the Chemistry and Biochemistry of Plant Lipids, Academic Press, London, pp 247–283
2. **Aspinall D, Paleg LG** (1981) Proline accumulation. Physiological aspects. *In* LG Paleg, Aspinall D, eds, Physiology and Biochemistry of Drought Resistance, Academic Press, Sydney, pp 206–241
3. **Bates LS, Waldren RP, Teare ID** (1973) Rapid determination of free proline for water-stress studies. Plant Soil **39:** 205–207
4. **Delseny M, Cooke R, Penon P** (1983) Sequence heterogeneity in radish ribosomal RNA genes. Plant Sci Lett **30:** 107–111
5. **Finkelstein RR, Tenbarge KM, Shumway JE, Crouch ML** (1985) Role of ABA in maturation of rapeseed embryos. Plant Physiol **78:** 630–636
6. **Finkelstein RR, Crouch ML** (1986) Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol **81:** 907–912
7. **Finkelstein RR, Somerville CR** (1989) Abscisic acid and high osmoticum promote accumulation of long chain fatty acids in developing embryos of *Brassica napus*. Plant Sci **61:** 213–217
8. **Haughn G, Somerville CR** (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet **204:** 430–434
9. **Hodgson CP, Fisk RZ** (1987) Hybridization probe size control: optimized "oligolabelling." Nucleic Acids Res **14:** 6295
10. **Hurkman WJ, Tanaka CK** (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol **81:** 802–806
11. **Jacobsen JV, Shaw DC** (1989) Heat-stable proteins and abscisic acid action in barley aleurone cells. Plant Physiol **91:** 1520–1526
12. **Karssen CM, Brinkhorst-van der Swan DLC, Breekland AE, Koornneef M** (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies of abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta **157:** 158–165
13. **King J, Laemmli UK** (1971) Polypeptides of the tail fibres of bacteriophage T4. J Mol Biol **62:** 465–473
14. **Koornneef M, Hanhart CJ** (1984) The localization of two ABA-insensitive genes. Arabid Inf Serv **21:** 5–9
15. **Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM** (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol **90:** 463–469
16. **Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM** (1982) The isolation of abscisic acid (ABA)-deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theoret Appl Genet **61:** 385–393
17. **Koornneef M, Reuling G, Karssen CM** (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. Physiol Plant **61:** 377–383
18. **Marre E** (1979) Fusicoccin: a tool in plant physiology. Ann Rev Plant Physiol **30:** 273–288
19. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497
20. **Pang PP, Pruitt RE, Meyerowitz EM** (1988) Molecular cloning, genomic organization, expression and evolution of 12S seed storage protein genes of *Arabidopsis thaliana*. Plant Mol Biol **11:** 805–820
21. **Simon AE, Tenbarge KM, Scofield SR, Finkelstein RR, Crouch ML** (1985) Nucleotide sequence of a cDNA clone of *Brassica napus* 12S storage protein shows homology with legumin from *Pisum sativum*. Plant Mol Biol **5:** 191–201
22. **Van Steveninck RFM, Van Steveninck ME** (1983) Abscisic acid and membrane transport. *In* FT Addicott, ed, Abscisic Acid. Praeger Publishers, New York, pp 171–236
23. **Zeevaart JAD, Creelman RA** (1988) Metabolism and physiology of abscisic acid. Ann Rev Plant Physiol Plant Mol Biol **39:** 439–473