

Regulation and Function of the Arabidopsis *ABA-insensitive4* Gene in Seed and Abscisic Acid Response Signaling Networks¹

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We have characterized developmental, environmental, and genetic regulation of *abscisic acid-insensitive (ABI4)* gene expression in Arabidopsis. Although expressed most strongly in seeds, *ABI4* transcripts are also present at low levels in vegetative tissue; vegetative expression is not induced by abscisic acid (ABA) or stress treatments. Comparison of transcript levels in mature seeds of ABA-insensitive, ABA-hypersensitive, ABA-deficient, or heterochronic mutants indicates that *ABI4* expression is altered in only two of the backgrounds, the ABA-insensitive mutants *abi1-1* and *abi3-1*. To determine whether *ABI4* is necessary and/or sufficient for ABA response, we assayed the effects of loss of *ABI4* function and ectopic *ABI4* expression on growth and gene expression. We examined genetic interactions among three ABA response loci, *ABI3*, *ABI4*, and *ABI5*, by comparing phenotypes of mutants, ectopic expression lines, mutants carrying an ectopically expressed transgene, and the corresponding wild-type lines. Our results indicate some cross-regulation of expression among *ABI3*, *ABI4*, and *ABI5* and suggest that they function in a combinatorial network, rather than a regulatory hierarchy, controlling seed development and ABA response.

Abscisic acid (ABA) regulates many aspects of plant growth and development, including embryogenesis, water relations, and tolerance of a variety of environmental stresses (for review, see Leung and Giraudat, 1998). Many lines of evidence indicate that there are multiple ABA perception and signaling mechanisms. Multiple receptor types are implicated by the variation in stereospecificity among ABA responses, studies of response to impermeant ABA-bovine serum albumin conjugates (Jeannette et al., 1999), and microinjection studies showing that ABA has both intra- and extra-cellular sites of action (for review, see Leung and Giraudat, 1998). Many likely signaling intermediates correlated with ABA response (e.g. ABA-activated or -induced kinases and DNA-binding proteins that specifically bind ABA responsive elements) have been identified by molecular and biochemical studies (for review, see Leung and Giraudat, 1998), but the relationships among these proteins are unclear. Genetic studies, especially in Arabidopsis, have identified many loci involved in ABA response and analyzed their functional roles

(for review, see Bonetta and McCourt, 1998; Leung and Giraudat, 1998).

The maize *viviparous 1 (vp1)* and Arabidopsis *ABA-insensitive (abi)* and *enhanced response to ABA (era)* mutants are the most extensively characterized ABA response mutants. The *vp1* mutations of maize have pleiotropic effects on seed development, not all of which can be ascribed to defects in ABA response (Robertson, 1955), suggesting that *VP1* mediates responses to a variety of signals including ABA. Four Arabidopsis loci affecting ABA sensitivity have also been extensively characterized: *ABI1*, *ABI2*, *ABI3*, and *ERA1* (for review, see Finkelstein and Zeevaert, 1994; Bonetta and McCourt, 1998). Mutations at these three *ABI* loci result in reduced seed dormancy and a reduction in sensitivity to exogenous ABA for inhibition of germination. In contrast, the *era1* mutants have increased dormancy and sensitivity to exogenous ABA for inhibition of seedling root growth and induction of stomatal closing (Cutler et al., 1996; Pei et al., 1998). Mutants at two additional Arabidopsis loci, *ABI4* and *ABI5*, were also selected for their ability to germinate on ABA concentrations inhibitory to wild-type germination (Finkelstein, 1994). Initial physiological and genetic analyses suggested that the *ABI3*, *ABI4*, and *ABI5* loci were likely to be acting in the same seed-specific signaling pathway; mutants at all three loci exhibited defects in seed ABA sensitivity and accumulation of at least one late embryogenesis abundant transcript, but displayed normal vegetative growth (Finkelstein and Somerville, 1990; Finkelstein, 1994). Furthermore, in studies of digenic mutants, mutations at all three loci greatly enhanced the

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ABA-resistance of *abi1* mutants and mutations in the *ABI3* and *ABI5* loci also significantly enhanced the ABA-resistance of *abi2* mutants. In contrast, combination of a weak *abi3* allele (*abi3-1*) with either *abi4-1* or *abi5-1* resulted in only slightly greater resistance. Prior to a molecular description of the *abi4-1* and *abi5-1* mutations it was not possible to determine the likely order of gene action in this proposed pathway because the *abi4* and *abi5* mutations might have been either null or leaky mutations. In fact, the observation that *abi4-1* and *abi5-1* mutant seeds were desiccation tolerant and only slightly resistant to ABA (Finkelstein, 1994), whereas severe *abi3* alleles resulted in production of desiccation intolerant highly ABA-insensitive "green seeds" (Nambara et al., 1992), lent support to the view that the *abi4-1* and *abi5-1* mutations were leaky. However, we have now cloned *ABI4* and *ABI5* and found that the *abi4-1* and *abi5-1* alleles have very severe mutations (Finkelstein et al., 1998; Finkelstein and Lynch, 2000).

The *ABI3*, *ABI4*, and *ABI5* genes have been cloned and found to encode putative transcription factors of the B3 domain, AP2 domain, and bZIP factor classes, respectively (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). Although mutations in *ABI3*, *ABI4*, and *ABI5* have their greatest impact on gene expression during seed maturation, all three genes are expressed to a limited degree in vegetative tissues (Finkelstein et al., 1998; Rohde et al., 1999; Finkelstein and Lynch, 2000), suggesting they may play a role in vegetative ABA response. Consistent with this, additional *abi4* mutants have recently been isolated on the basis of exhibiting salt-resistant germination (Quesada et al., 2000) or sugar-insensitive seedling growth (Gibson et al., 1999; Huijser et al., 1999; Arenas-Huertero et al., 2000).

Ectopic expression of *ABI3* has been shown to confer ABA-inducible vegetative expression of several "seed-specific" genes, leading to the suggestion that *ABI3* expression is sufficient for the seed developmental program and ABA sensitivity. However, changes in seed ABA sensitivity during embryogenesis cannot be explained by fluctuations in *ABI3* content, which remains relatively constant (on a per cell basis) through embryogenesis (Parcy et al., 1994). This indicates that although required for ABA response, *ABI3* content is not the only factor determining the degree of ABA sensitivity for any specific response.

In addition to the genetically defined transcription factors involved in seed and ABA response described above, many factors presumed to regulate ABA-inducible and embryonic gene expression have been identified biochemically (for review, see Leung and Giraudat, 1998). Although it is likely that many of these transcription factors regulate some of the same genes, the majority of specific target genes for most regulatory factors are unknown. Furthermore, for most factors it is not known whether regulation of

common target genes is accomplished by independent binding to distinct cis-acting sites, activation of a regulatory cascade, combinatorial action of factors, or a combination of these mechanisms. Many of these questions can be addressed by a molecular analysis of lines with loss or gain of specific regulatory factors, resulting from mutations or ectopic expression.

In the present study we have characterized the developmental, environmental, and genetic regulation of *ABI4* expression. In addition, we have compared different *abi4* mutant alleles, *ABI4* ectopic expression lines, and wild-type plants to assay the role of *ABI4* function in regulating gene expression in embryos and seedlings, cold, saline, and osmotic stress sensitivity, and sensitivity to ABA inhibition of root growth. We have also tested whether the previously described "hypersensitivity" to ABA due to ectopic *ABI3* expression is dependent on *ABI4* or *ABI5* function. Our results indicate some cross-regulation among these *ABI* loci, but the observed alterations in gene expression in the mutants are more consistent with combinatorial control by these three loci than a simple linear pathway.

RESULTS

Function of the *ABI4* Protein and Its Mutant Alleles

Molecular cloning of the *ABI4* gene identified it as a member of the *APETALA2* domain family and thus presumed to encode a transcriptional regulator. Many transcription factors have been shown to have transcription activation function in hybrid proteins expressed in heterologous systems such as yeast. To determine whether *ABI4* is capable of transcription activation in yeast we constructed a fusion with the *GAL4* DNA-binding domain (*GAL4*-BD) and tested its ability to activate a *GAL4*-responsive promoter in yeast. A fusion protein containing all but the first two and last amino acids of *ABI4* (residues 3–327) directed high level expression of a β -galactosidase reporter gene (Table I), consistent with function as a transcriptional activator.

The original *abi4-1* mutant allele contains a frameshift at codon 157 (of 328), resulting in a protein containing the presumed DNA binding and dimerization domains, but lacking the presumed activation domain(s). Additional mutant *abi4* alleles have recently been isolated in screens for sugar-insensitive (Gibson et al., 1999; Huijser et al., 1999; Arenas-Huertero et al., 2000) or salt-resistant (Quesada et al., 2000) seedling growth, leading Quesada et al. to suggest that the *abi4-1* mutant still had residual *ABI4* function. We have compared transcription activation function of full-length and truncated *ABI4* products in a one-hybrid assay in yeast. A fusion containing only amino acids 3 through 156 was no more active than the product of the vector alone (Table I). This indicates that the *abi4-1* encoded protein is inactive as

Table 1. Transcriptional activation by *GAL4BD-ABI4* fusions in yeast

Construct	ABI4 Amino Acids Present	Putative Activation Domains Present	β -Galactosidase Activity (Miller units) ^a
pGBD	None	None	5.2 \pm 2.9
pGBD-ABI4	3–327	Q-rich, P-rich, acidic	224.5 \pm 12.4
pGBD-ABI4 Bam	3–287	Q-rich, P-rich	66.7 \pm 0.1
pGBD-ABI4 Sac	3–224	Q-rich	42.5 \pm 4.8
pGBD-ABI4 Nla	3–156	None	6.8 \pm 4.4
pGBD-ABI4 C-term	157–327	Q-rich, P-rich, acidic	2.6 \pm 0.4

^a β -Galactosidase activity produced by activation of a *GAL7-lacZ* reporter gene by *GAL4BD* fusions containing various domains of *ABI4*. Data presented are averages \pm SD of assays with at least duplicate extracts.

a transcriptional activator in yeast, although it may still be capable of forming a DNA-binding complex.

The C-terminal half of the *ABI4* protein contains several regions that are candidates for transcription activation domains: a Gln-rich (amino acids 188–208), a Pro-rich (amino acids 275–289), and an acidic domain (amino acids 295–326). To determine whether any of these are required for transcription activation we have also tested fusions containing larger segments of *ABI4*. The longest of these contained all but the C-terminal acidic domain and its transcription activation function was reduced 5-fold relative to the full-length protein fusion. A fusion containing only the Q-rich domain had only approximately 18% the activity of the full-length fusion. These results suggest that all three putative activation domains contribute to activation function. Although necessary, the C-terminal portion is not sufficient; a fusion containing residues 157 through 327 does not activate reporter expression. However, we cannot rule out the possibility that the inactive fusions are misfolded or unstable.

As an independent test of *abi4-1* protein function we have compared an allelic series of *abi4* mutants with respect to their sensitivities to ABA inhibition of germination. In addition to *abi4-1* we tested alleles containing nonsense mutations such that the proteins are truncated at amino acids 193, 80, and 39 (in the *abi4-101* [*sis5-1*], *abi4-102* [*sis5-2*], and *abi4-103* [*sis5-3*] alleles, respectively). Thus the *abi4-103* mutant (Laby et al., 2000) is truncated five amino acids earlier than the frame-shift in *abi4-2* (*san5*) (Quesada et al., 2000), lacks the entire AP2 domain, as well as the presumed activation domain, and is therefore the most likely allele to produce a biochemical null. Comparison of ABA sensitivities for germination inhibition shows no statistically significant difference among these alleles (Fig. 1). Similar germination percentages were obtained with *ABI4* anti-sense transgenes in wild-type and *abi4-1* mutant backgrounds, although the lines containing an antisense transgene and the mutation germinated slightly faster (data not shown). However, the fact that the *abi4-1* and *abi4-103* mutants show equivalent sensitivities to ABA suggests that neither product has significant residual activity.

Regulation of *ABI4* Expression

Our initial characterization of *ABI4* expression indicated that transcript levels were highest in pooled stages of developing siliques, but reduced in the *abi4-1* mutant. In addition, very low *ABI4* transcript levels were detected in 11-d-old plants, indicating that expression was not seed specific (Finkelstein et al., 1998). We have now expanded our analysis to include developmental, environmental, and genetic regulation of *ABI4* expression. We have used a combination of RNA gel-blot analyses and assays of β -glucuronidase (*GUS*) activity derived from a variety of *ABI4* promoter-*GUS* fusions. These studies confirmed that expression is highest in seeds, with transcript levels fairly stable through the latter half of

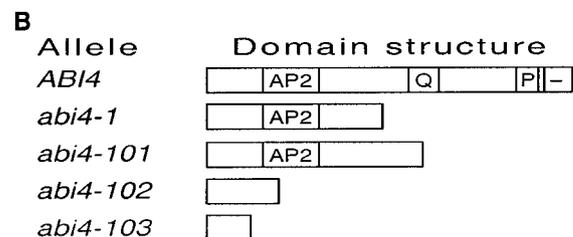
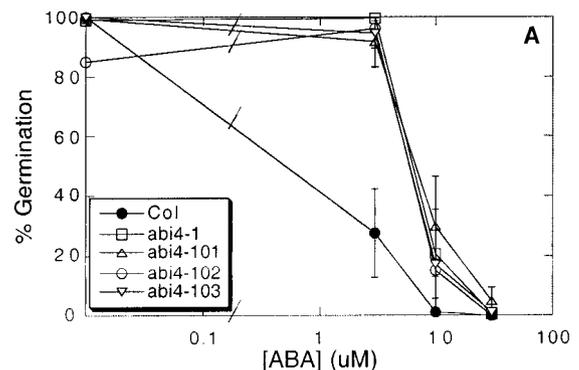


Figure 1. Comparison of sensitivities to ABA inhibition of germination for seeds of wild type and an *abi4* allelic series. Seeds were plated on the indicated concentrations of ABA and germination was scored after 7 d (A). Domain structures of these *abi4* alleles are shown in B. Data presented are averages \pm SD of at least duplicate assays.

embryogenesis, then increasing slightly at seed maturity (Fig. 2). We were surprised to find that *ABI4* transcript levels were quite similar in wild-type and *abi4-1* seeds. However, we had previously compared RNA from pooled stages of siliques and a slight bias toward dry seeds in the wild-type pool would have made the *ABI4* transcript appear more abundant in this sample.

Studies of GUS fusion lines indicate that the *ABI4* promoter is active from globular stage onward and its activity is limited to the embryo, where it is uniformly expressed (Fig. 3, A–C). Additional histochemical staining showed limited patches of *ABI4* promoter activity in vegetative tissue, especially in vascular tissue, consistent with the low levels of *ABI4* transcript observed after germination (Fig. 3, D–F). No significant change in GUS activity was detected in 5-d-, 11-d-, or 4-week-old plants subjected to a variety of stress (drought, saline, high Glc, or cold) or ABA treatments (data not shown). Although GUS activity was observed in mature anthers of transgenic lines (Fig. 3, G and H), but not in those of untransformed controls (data not shown), activity of the endogenous *ABI4* promoter was not confirmed by reverse transcriptase-PCR analyses of RNA isolated from floral buds (data not shown).

ABI4 is one of many genes with pleiotropic effects on seed development. To determine whether *ABI4* is likely to be part of a regulatory hierarchy with other known seed regulatory loci, we measured *ABI4* transcript levels in dry seeds of a variety of heterochronic (*fus3* and *lec1*), ABA response (*abi* and *era1*), or ABA biosynthetic (*aba1*) mutants. *ABI4* transcript accumulation was nearly normal in most of the genotypes tested, but was significantly reduced in *abi1-1* and *abi3-1* (Fig. 4). Although the *fus3* and *lec1* dry seeds are dead, even a slight reduction in *ABI4* transcript levels would be consistent with normal expression until viability is lost (at late embryogenesis stage).

Role of *ABI4* in Vegetative Growth Regulation

The similar, but relatively weak, ABA resistance of the *abi4* mutants suggests that *ABI4* plays a minor role

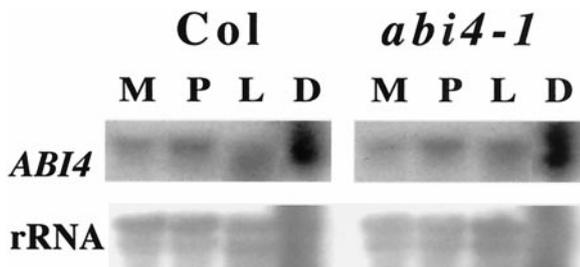


Figure 2. *ABI4* transcript accumulation during the latter half of embryogeny. RNA was isolated from Col or *abi4-1* siliques at 8 to 11 days post anthesis (dpa) (M, maturation), 12 to 16 dpa (P, post-abscission), 17 to 21 dpa (L, late embryogenesis), and >21 dpa (D, dry seeds) of each genotype. Each lane contains approximately 8 μ g of total RNA.

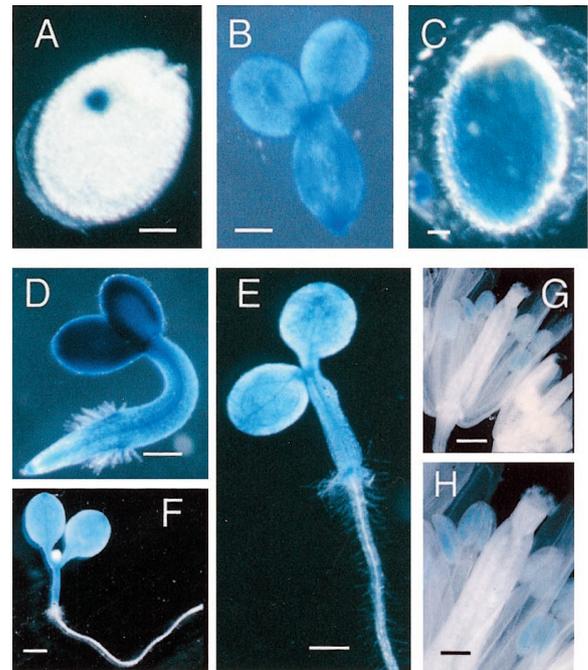


Figure 3. *ABI4*-driven GUS expression. Histochemical staining of globular embryo in seed (A), excised 5-d embryo (B), and mature seed (C); 2-d (D), 3-d (E), and 5-d (F) seedlings, and transgenic (G and H) flowers. Bar = 100 μ m in A and C; bar = 150 μ m in B; bar = 300 μ m in D; bar = 500 μ m in E and G; bar = 1 mm in F; bar = 250 μ m in H.

in ABA response or acts redundantly with other ABA response loci. To determine whether *ABI4* is necessary and/or sufficient for ABA response, we have assayed the effects of loss of *ABI4* function and ectopic *ABI4* expression on growth and gene expression.

The *abi4-1* mutant was isolated on the basis of ABA-resistant germination and our initial characterization suggested its effects were seed specific (Finkelstein, 1994). Upon cloning *ABI4* we found that it was expressed at low levels in vegetative tissue (Finkelstein et al., 1998). Furthermore, new mutant *abi4* alleles have been identified in seedling screens (Gibson et al., 1999; Huijser et al., 1999; Arenas-Huertero et al., 2000; Quesada et al., 2000), indicating that *ABI4* function is required during seedling growth under some conditions. To analyze the role of *ABI4* in regulation of vegetative growth, we compared the effects of loss of *ABI4* function and ectopic *ABI4* expression on two additional physiological responses: ABA sensitivity of root growth and growth under saline or osmotic stress. The ectopic expression lines analyzed had a broad range of *ABI4* expression levels and associated phenotypes.

Although loss of *ABI4* function had little effect on root growth, ectopic *ABI4* expression resulted in hypersensitivity to root growth inhibition by ABA (Fig. 5). Growth of the transgenic line with strongest *ABI4* expression (114A) was stunted on hormone-free medium (approximately 40% of wild type) and completely blocked by 3 μ M ABA, a concentration per-

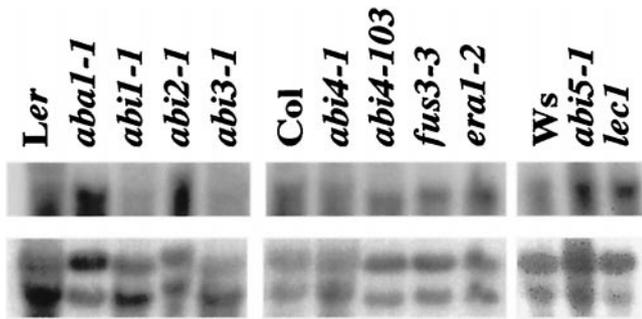


Figure 4. *ABI4* transcript accumulation in mature dry seeds of ABA response (*abi*), ABA biosynthetic (*aba*), or seed regulatory (*fus3*, *lec1*, and *era1*) mutants. Mutant lines are grouped according to their ecotype backgrounds along with the appropriate wild-type line. Each lane contains approximately 5 μ g of total RNA. Top row is *ABI4* transcript; bottom row is rRNA.

mitting approximately 35% of control growth in wild-type lines. The more weakly expressing transgenic lines also showed significantly less root growth than wild-type plants when exposed to ABA, but grew normally on hormone-free medium. For comparison, root growth in an ABA-hypersensitive line produced by ectopic constitutive expression of *ABI3* (*ABI3^c*) was completely blocked by 3 μ M ABA, but was normal on hormone-free medium. In contrast to the consistent increase in ABA-sensitivity of root growth produced by ectopic *ABI4* expression, the *ABI4* transgenes had very limited effects on saline or osmotic stress sensitivity of plant growth (data not shown). These results show that increased *ABI4* function is sufficient for increased ABA sensitivity of vegetative tissues, but has negligible effects on stress tolerance.

ABI4-Regulated Gene Expression

Our initial characterization of the *abi4-1* mutant showed that *AtEm6* transcript accumulation was reduced in dry seeds (Finkelstein, 1994). We have extended this analysis to include eight additional genes whose expression was assayed through the latter half of embryogenesis. These results show that the *abi4-1* mutant has only minor effects on expression of most of these genes (Fig. 6). Although there is a substantial reduction in dry seed transcript levels of *AtEm6* and the *LeaD34* homolog, *AtEm1* expression is only slightly reduced and some of these transcripts (e.g. *AtEm1* and *AtEm6*) appear to accumulate earlier in the mutant than in wild-type seeds. The storage protein genes, *At2S3* and *CRC*, also show only minimal changes in expression (less than 25% reduction) in the *abi4-1* mutant, whereas accumulation of other transcripts (e.g. *RAB18* and the *vicilin* homolog) is essentially unaffected in this mutant (within 10% of the wild type). In contrast, expression of some genes (*M17* and mid-embryogenesis stage expression of the oleosin homolog) is increased in the *abi4-1* mutant, as

observed for the *abi5-1* mutant (Finkelstein and Lynch, 2000), rather than decreased as in the *abi3* mutants (Parcy et al., 1994). Several of the *LEA* genes are ABA-inducible in vegetative tissues, but *ABI4* is required for only a subset of these (*AtEm1* and *AtEm6*) to be fully expressed (Fig. 7). The relatively minor effects of the *abi4-1* mutation on transcript accumulation indicate that *ABI4* is not the major regulator of embryonic expression for most of these genes, but that it does play a role in seedling and vegetative gene regulation.

To test whether *ABI4* is sufficient for expression of any of these genes we measured their transcript levels in five independent transgenic lines ectopically overexpressing *ABI4*, designated *ABI4^c* (Fig. 8). Ectopic *ABI3* expression had previously been shown to confer ABA-inducible expression of several “seed-specific” storage protein and *lea* genes in vegetative tissues (Parcy et al., 1994); therefore, we included a line with a strongly expressed *ABI3* transgene, designated *ABI3^c*, for comparison. The *ABI4^c* transgenic lines displayed a broad range of *ABI4* expression and many of them showed ABA-inducible vegetative expression of the same “seed-specific” genes as in the *ABI3* ectopic expression lines. In addition, constitutive expression of *ABI4* resulted in ABA-inducible *ABI3* expression and hyperinduction of *ABI5*. As previously shown for *ABI3* transgenic lines (Parcy et al., 1994), there was not a simple correlation between the level of expression for any of the *ABI* genes and the magnitude of target gene induction. Furthermore, the importance of any given regulator varied depending on the target gene. For example, *AtEm1* and *AtEm6* induction correlated fairly well with *ABI5* expression, consistent with their strong dependence on *ABI5* function for

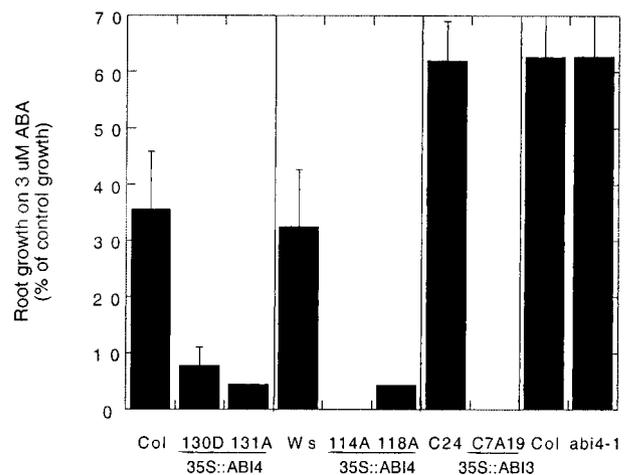


Figure 5. Effects of *ABI4* or *ABI3* function on ABA sensitivity of root growth. After 2 d of growth on hormone-free medium, seedlings were transferred to fresh media containing 0 or 3 μ M ABA. New growth was measured after 4 d and expressed as a percentage of growth on the control medium. Each set represents the average of at least 10 replicates within an individual experiment; standard deviations are expressed as percentages of the control growth.

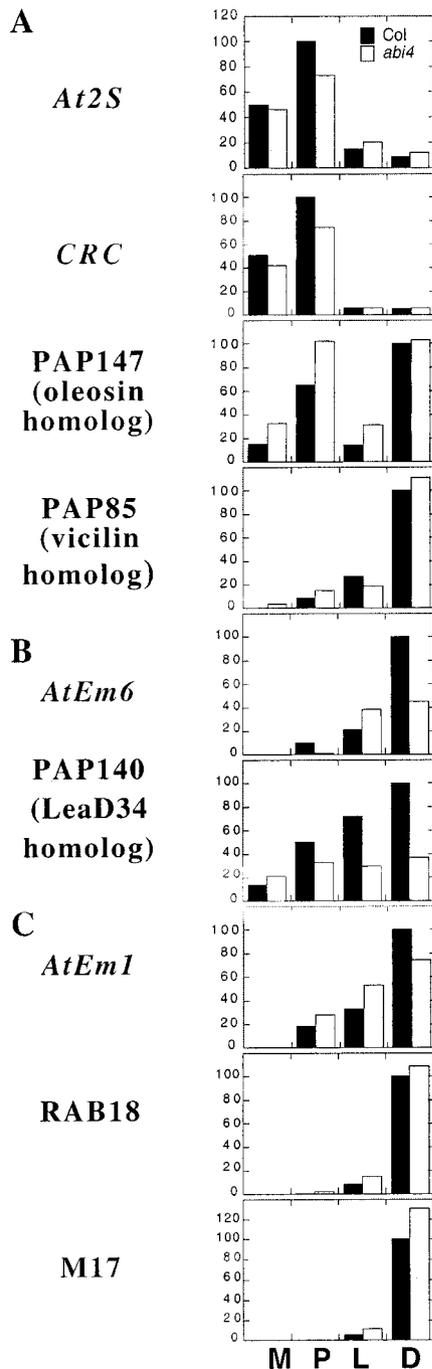


Figure 6. Comparison of transcript accumulation in developing siliques of wild-type versus *abi4-1* mutants. Relative abundance of MAT (A), LEA (B), and LEA-A (C) class transcripts from four developmental stages (M, P, L, and D; stages as defined in Fig. 2) is shown for Col and *abi4*, with peak level in wild type arbitrarily set at 100. Hybridization to the indicated probes was quantified from RNA gel blots by either phosphorimaging or densitometry of autoradiograms, then normalized relative to total RNA quantified by phosphorimaging of rRNA hybridization.

expression in seeds (Finkelstein and Lynch, 2000). In contrast, the storage protein genes *At2S3* and *CRC* were far more strongly induced by the *ABI3^c* trans-

gene than by the *ABI4^c* transgenes, consistent with their strong dependence on *ABI3* for embryonic expression. However, even the highest *ABI3* transcript levels in the *ABI4^c* lines were approximately 100-fold lower than in the *ABI3^c* line and were therefore unlikely to be sufficient for promoting the observed level of storage protein gene expression. It is possible that the *ABI4^c* expression potentiates the effects of *ABI3* and *ABI5* in promoting *At2S3* and *CRC* expression. The differential response of the storage protein and lea genes to *ABI4^c* expression alternatively might reflect either ecotype or transgene differences since lines 114 and 118 are in a different genetic background and have a much shorter 5'-untranslated region (UTR) than lines 130 and 131. However, regardless of the magnitude of response in individual transgenic lines, these results indicate that ectopic expression of *ABI3* or *ABI4* is sufficient for ABA-inducible vegetative expression of some *LEA* and storage protein genes and suggest that the transgenes' effects may be partially mediated by *ABI5*.

Cross-Regulation of *ABI3*, *ABI4*, and *ABI5* Function and Expression

To determine whether the effects of ectopic *ABI3* expression were dependent on *ABI4* or *ABI5* function, a strongly expressed *ABI3^c* transgene was backcrossed into *abi4-1* and *abi5-1* mutant backgrounds. Because the transgene and the *abi* mutations were all in different genetic backgrounds, multiple segregants were analyzed to ensure that we were not monitoring random segregation of unknown modifying genes in the various backgrounds. Although *ABI3* expression was controlled by a constitutive doubly enhanced cauliflower mosaic virus (CaMV)35S promoter, *ABI3* transcript accumulation varied among segregants

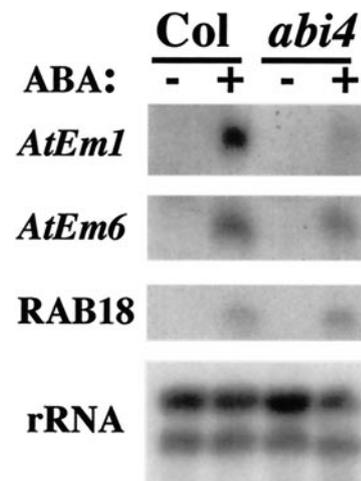


Figure 7. RNA gel-blot comparison of ABA-inducible LEA and LEA-A transcript accumulation in 13-d plantlets of wild-type versus *abi4-1* mutants. RNA was extracted from 13-d seedlings incubated for 2 d on plates $\pm 50 \mu\text{M}$ ABA, then analyzed by RNA gel blots hybridized to cloned probes for the indicated transcripts.

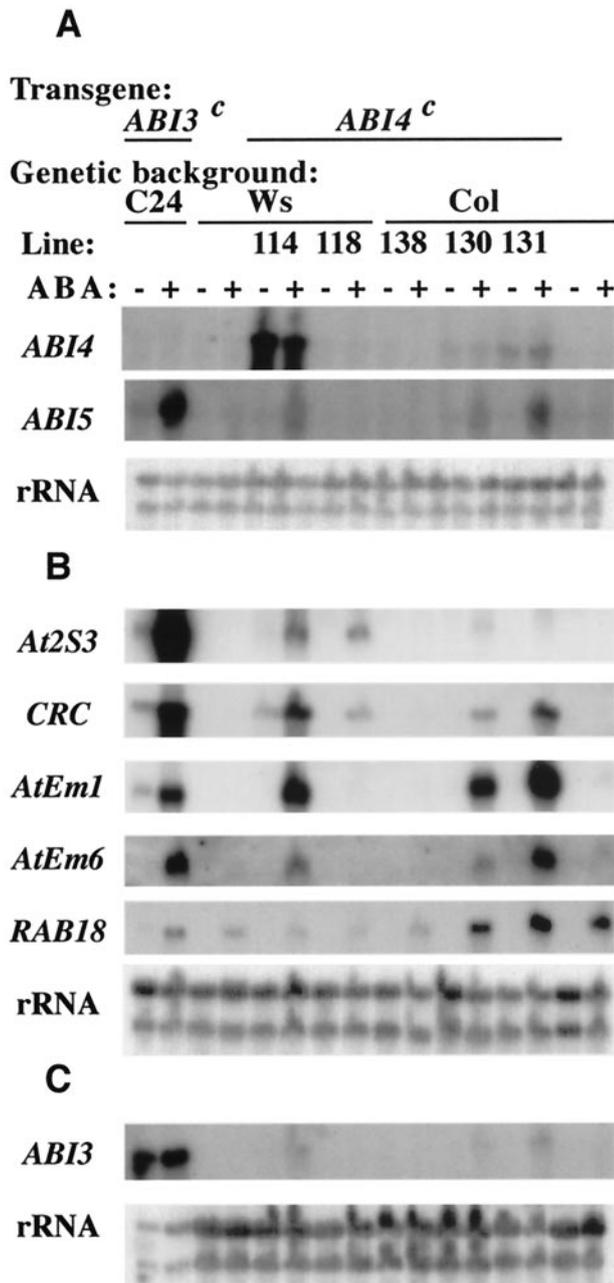


Figure 8. Effects of ectopic *ABI4* expression on ABA-inducible vegetative accumulation of transcripts normally expressed primarily in seeds. RNA was extracted from 13-d seedlings incubated for 2 d on plates $\pm 50 \mu\text{M}$ ABA, then analyzed by RNA gel blots hybridized to cloned probes for the indicated transcripts. A, All lanes contain 10 μg of total RNA. B, All lanes contain 5 μg of RNA. C, Lanes with RNA from *ABI3* ectopic expression line contain 1 μg of RNA; all others contain 10 μg of RNA.

and marker gene expression correlated fairly well with the level of *ABI3* transcript in the ABA-treated plants (Fig. 9). However, in the *abi5* line with *ABI3* expression comparable with that in the wild-type (C24) background, *At2S3*, *CRC*, *AtEm6*, and *RAB18* expression appear slightly reduced. These results suggest that although all three *ABI* genes monitored

may promote ABA-inducible vegetative expression of the storage protein and lea genes, strong expression of one (e.g. *ABI3*) can reduce reliance on the others. We do not yet know whether ectopic *ABI5* expression has similar effects on ABA-inducible responses, or if *ABI3* function is required for *ABI4^c* expression to be effective.

RNA gel-blot analyses have provided evidence of cross-regulation among the *ABI* loci. *ABI5* expression is reduced to varying extents in *aba1-1*, *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1*, and *abi5-1* mutant seeds (Finkelstein and Lynch, 2000) and hyperinduced by ABA in transgenic plants ectopically expressing either *ABI3* or *ABI4* (Fig. 8). *ABI4* expression is reduced in *abi1-1* and *abi3-1* seeds (Fig. 4). *ABI3* expression is induced by ABA in *ABI4^c* lines (Fig. 8). To determine whether *ABI3* expression in seeds is regulated by *ABI4* or *ABI5* we assayed activity of an *ABI3::GUS* transgene, as well as transcript accumulation. *ABI3* promoter activity was reduced approximately 3-fold in *abi5-1* seeds and 4-fold in *abi4-1* seeds (Table II). *ABI3* transcript levels are slightly reduced in *abi4-1* mutant seeds, but appear similar in *abi5-1* and Wassilewskija (Ws) (wild-type) seeds (Fig. 10). These results indicate that, whereas *ABI4* and *ABI5* may regulate *ABI3* promoter activity, *ABI3* transcript accumulation is controlled by additional factors, possibly including mRNA stability.

DISCUSSION

ABI4 Is a Transcriptional Activator

ABI4 is a member of the APETALA2 (AP2) domain family of transcriptional regulators. Sequence comparisons with other family members show extremely limited homology outside the conserved AP2 domain, which has been shown to be involved in DNA binding (Ohme-Takagi and Shinshi, 1995) and has been suggested to participate in dimerization of other AP2 domain proteins (Okamoto et al., 1997). Although transcription activation functions have been demonstrated for other family members (Buttner and Singh, 1997; Stockinger et al., 1997), it was not clear whether *ABI4* alone could activate transcription. Assays of reporter gene expression have demonstrated that *ABI4* does supply a strong activation function to a fusion with the GAL4-BD. In contrast, fusions with a series of truncated portions of *ABI4* have little or no activity in this assay. These results indicate that loss of the C-terminal acidic domain and the adjacent Pro-rich domain severely disrupt the transcription activation function of *ABI4*. Both of these domains are missing in all of the available mutant alleles.

The fact that severe mutations in the *ABI4* gene produce relatively weak phenotypic effects suggests that defects in *ABI4* function may be masked by genetic redundancy. Although no close *ABI4* homologs have been reported, we have cloned a gene corresponding to the most strongly hybridizing frag-

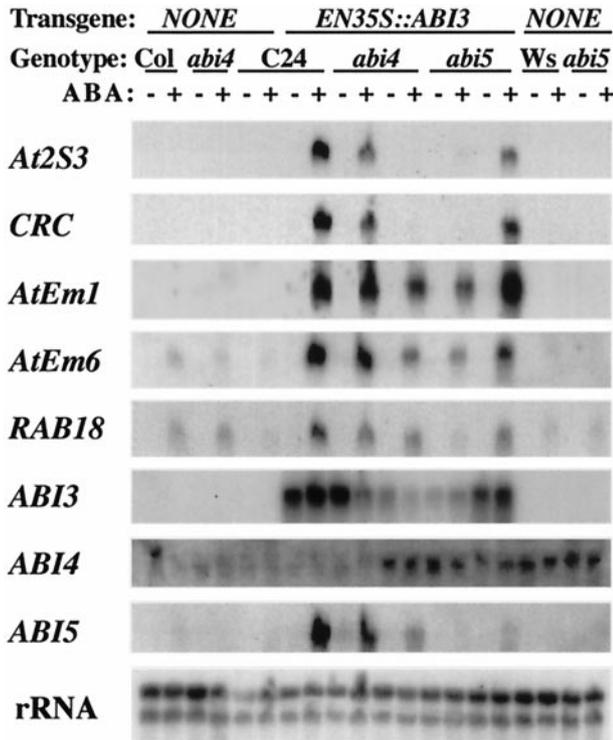


Figure 9. Effects of the *abi4-1* and *abi5-1* mutations on ABA-induced, *EN35S::ABI3*-dependent vegetative accumulation of primarily seed-expressed transcripts. RNA was extracted from 13-d seedlings incubated for 2 d on plates $\pm 50 \mu\text{M}$ ABA, then analyzed by northern blots hybridized to cloned probes for the indicated transcripts. Ten genotypes were compared: Col, *abi4-1*, Ws, *abi5-1*, and C24 with no transgenes, a strongly expressed *EN35S::ABI3* transgene in the C24 background, and two independent *EN35S::ABI3* transgenic segregants in each of the *abi4-1* and *abi5-1* backgrounds, illustrating the range of transgene and marker gene expression observed among the segregants.

ment detected by reduced stringency genomic DNA gel blots (data not shown). The predicted products of this gene and the closest match to *ABI4* currently available in the database are highly similar to *ABI4* within the AP2 domain (54% and 60% identical in predicted amino acid sequence, respectively). However, they are very divergent from *ABI4* outside this domain and cluster more closely with the DREB2 subfamily (Liu et al., 1998) in multiple sequence alignments. Neither has been associated with a mutant phenotype and it is not known whether either plays a role in ABA signaling.

Seed Sensitivity to ABA Shows Little Variation among *abi4* Alleles

Mutant *abi4* alleles have been identified in a variety of screens including ABA-resistant germination (Finkelstein, 1994), salt-resistant germination (Quesada et al., 2000), and sugar-resistant seedling growth (Gibson et al., 1999; Huijser et al., 1999; Arenas-Huertero et al., 2000).

Comparison of sensitivities to ABA inhibition of germination among these mutants has produced a surprising range of results. Although the *abi4-2* mutant was described as germinating almost completely on $30 \mu\text{M}$ ABA (Quesada et al., 2000), we found that all of the other alleles were blocked from germinating by this ABA concentration. All were approximately 3-fold less resistant to ABA than was reported for *abi4-2*, including a mutant producing a more severely truncated ABI4 protein than that in the *abi4-2* mutant. It is not clear why the *abi4-2* mutant appeared so much more resistant to ABA than any of the other alleles, but this result cannot be simply ascribed to the relative lengths of the mutant gene products.

ABI4 Function in Seeds

A combination of RNA gel-blot analyses and measurements of GUS activity driven by an *ABI4* promoter fragment indicate that *ABI4* is expressed throughout the embryo, from globular stage onward, but transcript levels are highest at seed maturity. Consistent with this expression pattern, the most severe defects of the mutants are observed in mature seeds: decreased sensitivity to ABA, salt and osmotic inhibition of germination, and significantly reduced expression of some leaf genes. Although there are some alterations in transcript accumulation for genes expressed at earlier stages, most of these are relatively subtle changes, suggesting that *ABI4* plays only a minor role in regulating their expression. It is interesting that some of the transcripts (e.g. M17) show increased accumulation, indicating that *ABI4* may function as an activator or a repressor of different subsets of genes. Similar results have been described for regulation by *ABI5* (Finkelstein and Lynch, 2000). Although M17 is expressed at high levels in late embryogenesis, its expression is repressed in young seedlings by ABA, salt, or drought treatment (Raynal et al., 1999). It is possible that *ABI3* is essential for its induction late in embryogenesis, whereas *ABI4* and *ABI5* mediate the repression in response to ABA and stresses.

ABI4 expression is near normal in seeds of a variety of ABA-insensitive, ABA-hypersensitive, ABA-deficient, and heterochronic mutants, but slightly reduced in the *abi1-1* and *abi3-1* mutants. This contrasts with control of *ABI5* expression, which is regulated

Table II. *ABI3* promoter activity in *abi4* and *abi5* mutants

Genotype	GUS Activity (units/seed) ^a
Wild type (C24)	8,565 \pm 297
<i>abi4-1</i>	1,892 \pm 227
<i>abi5-1</i>	3,109 \pm 907

^a GUS activity in mature seeds carrying an *ABI3::GUS* transgene in wild-type (C24), *abi4*, and *abi5-1* mutant backgrounds. Data presented are averages \pm SD of assays with at least two independent extracts (C24) or segregants per genotype in the mutant backgrounds.

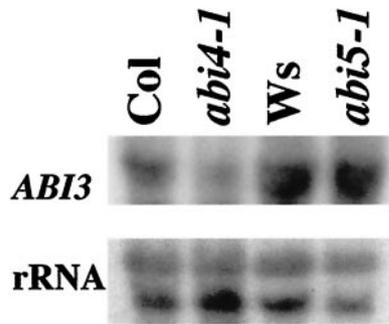


Figure 10. *ABI3* expression in *abi4-1* and *abi5-1* seeds. RNA gel-blot analysis of *ABI3* transcript accumulation in *abi4-1* and *abi5-1* seeds relative to their wild-type progenitors. Each lane contains 5 μ g of total RNA.

by ABA and all of the *ABI* loci (Finkelstein and Lynch, 2000). Although *ABI4* transcript accumulation is not regulated by many of these ABA- or seed response loci, it is still possible that *ABI4* protein accumulation or activity is dependent on some of these loci.

Role of *ABI4* in Post-Germinative Growth

ABI4 is expressed constitutively, but at very low levels, in vegetative tissues. Histochemical staining of *ABI4*-driven GUS activity shows low expression throughout the hypocotyl, roots, and cotyledons of young seedlings, which may be residual from the comparatively high expression levels in embryogenesis. Additional expression is observed in newly formed vegetative organs, but the localization patterns are variable and do not correlate with any specific tissues or structures.

Although previously described as ABA-inducible in 4-week-old plants (Wang et al., 1999), we saw no difference in *ABI4*-driven GUS expression in 5-d-, 11-d-, or 4-week-old plants treated with or without 100 μ M ABA. Consistent with this, we saw no effect of ABA treatment on *ABI4* transcript accumulation in 11-d-old plants. We also saw no difference in *ABI4*-driven GUS expression between control-treated plants and those exposed to drought, saline, or cold stresses. Although *ABI4* expression was recently reported to be "Glc-induced," based on reverse transcriptase-PCR comparison of transcript levels in plants grown for 15 d with or without 7% (w/v) Glc (Arenas-Huertero et al., 2000), *ABI4*-driven GUS activity shows no effect of 24 to 48 h exposure to 7% (w/v) Glc in 1- or 2-week-old plants. It is quite likely that the reported "Glc induction" reflects residual *ABI4* transcript in wild-type plants whose development is arrested by exposure to high exogenous Glc.

Although *ABI4* expression is not regulated by assorted stresses, *ABI4* function appears to be important for controlling a number of stress responses in young plants, including suppression of growth and induction of anthocyanin synthesis. Although *abi4*

mutants appear "tolerant" of salt, osmotic, or sugar stresses in germination and early seedling growth, this does not result in improved growth (i.e. weight gain) relative to wild type over long term exposure to saline or osmotic stresses.

Ectopic *ABI4* expression also resulted in hypersensitivity to ABA-inhibition of root growth. In fact, the lines with greatest *ABI4* expression had stunted root growth even in the absence of applied ABA, possibly due to excessive response to their endogenous ABA levels. These plants were stunted overall, presumably due to the limited surface area of their roots and consequent limitations of nutrient and water uptake.

Interactions among *ABI3*, *ABI4*, and *ABI5* in a Regulatory Network

Comparisons of embryonic gene expression in severe mutants of *ABI3*, *ABI4*, and *ABI5* have shown that these transcription factors regulate many of the same genes, but in different ways. In general, loss of *ABI3* function results in extreme reductions of embryonic gene expression (Parcy et al., 1994). In contrast, loss of *ABI4* or *ABI5* function produces relatively minor changes in expression of many *ABI3*-regulated embryonic genes (Fig. 6; Finkelstein and Lynch, 2000). The genes showing significantly altered expression in *abi4* or *abi5* mutants may be positively or negatively regulated by *ABI4* and *ABI5*. Such differences in regulatory patterns are not consistent with action of these *ABI* genes in a simple regulatory hierarchy.

We have also examined interactions of these three *ABI* loci in controlling *LEA* and storage protein gene expression in vegetative tissues ectopically expressing either *ABI3* or *ABI4*. Ectopic expression of either confers ABA-inducible expression of a subset of these "seed-specific" genes in 11-d-old plants. In addition, both sets of transgenic lines show hyperinduction of *ABI5* expression in response to ABA treatment. Although the *ABI4* ectopic expression lines also induce *ABI3* expression when exposed to ABA, this regulation is not reciprocated: *ABI4* transcript levels are unchanged by the presence of the *ABI3* transgene and/or ABA exposure. These results suggested that *ABI5* might be an important mediator of these transgenes' effects. However, comparison of ectopic *ABI3*-dependent marker gene expression in wild-type versus *abi5-1* backgrounds showed that *ABI5* function was not required for strong induction of the marker genes, although in some cases it was needed for full induction.

Attempts to correlate the degree of marker gene expression with any of the *ABI* genes indicated that different markers showed differential dependence on the *ABI* genes, but that strong expression of one *ABI* gene could reduce reliance on the others. To date, all of our analyses have focused on correlations among transcript levels and it is possible that these may not reflect the level of active protein for each of the *ABI*

genes. It is also possible that the relatively subtle phenotypic effects of severe biochemical lesions in *ABI4* and *ABI5* reflect genetic redundancy. *ABI5* is a member of a bZIP subfamily that includes at least four genes involved in ABA response that are expressed in young seedlings (Choi et al., 2000); these other family members may maintain *ABI3*-dependent vegetative expression of the “seed-specific” genes in an *abi5-1* background. No close *ABI4* homologs have been reported and we do not know whether any of the weakly related homologs play a role in ABA signaling.

In summary, initial studies of genetic interactions indicated that *ABI3*, *ABI4*, and *ABI5* were likely to act in a common seed-specific signaling pathway (Finkelstein, 1994) that could be introduced into vegetative tissue by ectopic expression of *ABI3* (Parcy et al., 1994). It was subsequently shown that none of these three loci exhibited truly seed-specific expression (Finkelstein et al., 1998; Rohde et al., 1999; Finkelstein and Lynch, 2000), consistent with the possibility that the effects of ectopic *ABI3* expression were dependent on either endogenous or induced expression of *ABI4* and/or *ABI5*. We have now shown that *ABI3* and *ABI5* cross-regulate one another and that ectopic *ABI4* expression confers ABA-inducible expression of *ABI3* and *ABI5*. *ABI3*, *ABI4*, and *ABI5* are all expressed throughout seed development and it is quite possible that expression of all three is regulated by as yet unidentified seed-specific regulators. In addition to the cross-regulation of *ABI3*, *ABI4*, and *ABI5* loci described above, *ABI1* and *ABI2* appear to auto- and inter-regulate each other (Leung et al., 1997). Furthermore, the effects of ectopic *ABI3* expression have been found to depend partially on *ABI1* function (Parcy and Giraudat, 1997). A hypothetical model depicting the observed cross-regulation among the *ABI* loci is shown in Figure 11.

Although the ectopic expression studies show that abnormally high expression of *ABI3* or *ABI4* is sufficient for ABA hypersensitivity, this may not accurately reflect their regulatory roles in wild-type plants. These results are consistent with the hypothesis that *ABI3*, *ABI4*, and *ABI5* act in a combinatorial network, each making differential contributions to regulation of genes expressed in seeds, and in response to ABA or assorted stresses. Direct physical interactions between rice homologs of *ABI3* and *ABI5* have recently been shown to enhance ABA-regulated gene expression in protoplasts derived from rice suspension-cultured cells (Hobo et al., 1999). This network is also likely to include additional genes already demonstrated to interact with members of this network, e.g. *LEC1* and *FUS3* (Parcy et al., 1997), and possibly functionally redundant genes, such as other members of the *ABI5*-related bZIP subfamily. Various subsets of this network could participate in

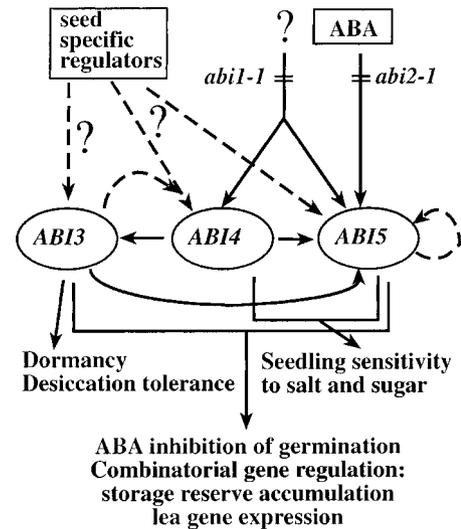


Figure 11. Proposed model of regulatory interactions among the *ABI* loci, ABA, and unidentified seed-specific regulators. Solid arrows represent cross-regulation among *ABI* loci demonstrated by reduced expression in mutants and/or increased expression in ectopic expression lines. Dashed arrows indicate speculative autoregulation (e.g. *ABI5*), regulation by unknown seed-specific factors, or stage-specific cross-regulation (e.g. full *ABI4* expression requires *ABI3* function in seeds, but is not induced by ectopic *ABI3* expression in vegetative tissues). Subsets of responses regulated by one or more *ABI* loci are listed at the bottom.

“enhancesome” formation regulating expression of individual genes.

MATERIALS AND METHODS

Plant Material

The *abi4-1* and *abi5-1* mutant lines were isolated from the Columbia (Col) and Ws backgrounds, respectively, as described in Finkelstein (1994). The *abi4-101*, *abi4-102*, and *abi4-103* mutants were isolated as sugar insensitive (*sis*) mutants in the Col background, as described in Laby et al. (2000). The *aba1-1*, *abi1-1*, *abi2-1*, and *abi3-1* mutant lines were isolated from the *Ler* background, as described in Koornneef et al. (1982, 1984). The *fus3-3* and *era1-2* mutants were isolated from the Col background, as described in Keith et al. (1994) and Cutler et al. (1996). The *lec1-1* mutant was isolated from the Ws background, as described in West et al. (1994).

The 35S::*ABI4* transgenic lines were constructed in the Col and Ws backgrounds by vacuum infiltration (Bechtold et al., 1993) with *Agrobacterium tumefaciens* carrying the plasmids described below. Lines 114, 118, and 138 contain a transgene with a 53-bp 5'-UTR, whereas lines 130 and 131 contain 0.5-kb of 5'-UTR between the CaMV35S promoter and the initiating ATG. *ABI4*::*GUS* lines were constructed in the Col background.

The *EN35S*::*ABI3* (isolate C7A19) and *ABI3*::*GUS* (isolate CAG3-11) transgenic lines were constructed in the C24 background as described in Parcy et al. (1994). To obtain lines with a *EN35S*::*ABI3* or *ABI3*::*GUS* transgene in the

mutant backgrounds, recombinant lines of *abi4-1* and *abi5-1* carrying the closely linked *pyrimidine-requiring* (*py*) mutation were crossed to the transgenic lines. Kanamycin-resistant F₂ individuals were selected and screened for thiamine auxotrophy resulting from the *py* mutation. Families homozygous for the transgene were identified as 100% kanamycin resistant in the F₃ generation.

For RNA isolation from siliques, plants were grown in soil in continuous light at 22°C. Siliques were harvested in pools corresponding to four developmental stages: maturation (8–11 dpa), post-abscission (12–16 dpa), late embryogenesis (17–21 dpa), and dry seed (>21 dpa). Seedlings were grown aseptically on Murashige-Skoog medium (1962) with 1% (w/v) Suc and 0.55% (w/v) agar for 11 d at 22°C in continuous light (50–70 $\mu\text{E m}^{-2} \text{s}^{-1}$) then transferred to fresh Murashige-Skoog medium with 1% (w/v) Suc, 0.7% (w/v) agar, and 0 or 50 μM ABA for an additional 2 d before harvest. All tissues for RNA isolation were weighed, flash frozen in liquid nitrogen, and stored at –70°C until extraction.

For histochemical staining of GUS activity in 4-week-old plants, flowers, and developing seeds, plants were grown in soil in continuous light at 22°C. To test for induction of *ABI4* promoter activity by ABA, 4-week-old plants were sprayed to run-off with 0.02% (w/v) Triton X-100, with or without 100 μM ABA, then tented with plastic wrap. Plants were sprayed once per day over 2 d, then harvested 24 h after the last application. Saline stress was imposed by watering plants with 100 mM NaCl on the 1st d, followed by 150 mM NaCl on the 2nd d. Tissues were harvested for GUS staining 24 h after the last treatment. Cold stress was imposed by incubation for 2 d in a dimly illuminated cold room at 6°C. Drought stress was imposed by not watering the plants for 13 d. For GUS staining of younger plants, seedlings were grown aseptically on Murashige-Skoog medium with 1% (w/v) Suc for 5 or 11 d at 22°C in continuous light (50–70 $\mu\text{E m}^{-2} \text{s}^{-1}$), then transferred to fresh Murashige-Skoog medium with 1% (w/v) Suc and 0 or 100 μM ABA, 150 mM NaCl, or 500 mM sorbitol for an additional 2 d before harvest. To test for induction of *ABI4* promoter activity by cold, one set of seedlings on control media was placed at 6°C for 2 d. To test for Glc-inducibility of *ABI4* promoter activity, seedlings were grown on minimal medium (Haughn and Somerville, 1986) or 0.5 \times Murashige-Skoog medium with 0 or 1% (w/v) Suc (Murashige and Skoog, 1962) for 1 or 2 weeks, then transferred to fresh media with or without 7% (w/v) Glc for an additional 2 d before harvest.

Transgene Constructs

Ectopic *ABI4* expression transgenes were constructed in pGA643 (An et al., 1988) with varying lengths of 5'-UTR between the CaMV35S promoter and the initiating codon of *ABI4*. One construct fused an intact *SspI* genomic fragment into the *BglIII* site of pGA643, blunted by filling in the ends with the Klenow fragment of DNA polymerase I. The resulting construct contained 0.5 kb 5' to the initiating codon of *ABI4*, as well as the coding region and 420 bp 3' to the stop codon, including several possible polyadenylation

sites. The other construct was a PCR-generated derivative of this *SspI* fragment with the same 3' end, but only 53 bp of 5'-UTR.

Antisense *ABI4* transgenes were constructed by subcloning a *BglIII-BamHI* fragment spanning 1 kb of 5' sequence and 0.9 kb of coding sequence in reverse orientation into the *BglIII* site of pGA643, blunted as described above.

ABI4::GUS transgenes were constructed by subcloning a series of *ABI4* genomic fragments into pBI101.3. All of the fragments terminated at an *AluI* site near the start of the *ABI4* coding sequence, thereby creating translational fusions including the first four codons of *ABI4*. Although there was substantial quantitative variability among the transgenic lines, the qualitative expression patterns were very similar. The data presented displays results with the lines containing the largest promoter fragment, a 3-kb *EcoRI-AluI* fragment.

Binary plasmids carrying the transgenes were introduced into *A. tumefaciens* line GV3101 by direct transformation, followed by selection for growth on appropriate antibiotics (kanamycin for pBI101 derivatives, tetracycline and kanamycin for pGA643 derivatives).

One-Hybrid Assays of Transcription Activation Function

Fusions between varying portions of *ABI4* and the GAL4-BD were constructed in the pGBD-C1 vector (James et al., 1996). All used an *EcoRI* site present at the 5' end of a cDNA clone to make translational fusions starting at codon 3 of *ABI4*. The "full length" *ABI4* fusion terminated at an *EcoRI* site that truncates the final product by one amino acid. Restriction sites used for the additional truncation products and the extent of each clone are described in Table I. All gene fusions were transformed into the yeast cell line PJ69–4A using the Alkali-Cation yeast transformation kit, according to the manufacturers instructions (Bio101, Vista, CA). Quantitative assays of GAL4-BD driven β -galactosidase gene expression were performed as described at <http://www.fhcrc.org/~gottschling/Bgal.html>.

Germination Assays

For germination assays, 60 to 100 seeds per treatment were surface sterilized in 5% (w/v) hypochlorite and 0.02% (w/v) Triton X-100, then rinsed three to four times with sterile water before plating on minimal medium (Haughn and Somerville, 1986) containing 0.7% (w/v) agar and ABA (mixed isomers, Sigma, St. Louis) at 0, 3, 10, or 30 μM in 15 \times 100-mm Petri dishes. The dishes were incubated 3 d at 4°C to break any residual dormancy, then transferred to 22°C in continuous light; germination was scored daily.

Root Growth Assays

For root growth assays, seeds were surface sterilized as described above before plating on germination medium (Valvekens et al., 1988) containing 0.7% (w/v) agar. Petri

plates were incubated 3 d at 4°C, then transferred to 22°C in continuous light (50–70 $\mu\text{E m}^{-2} \text{s}^{-1}$). After 2 d, germinated seedlings were transferred to germination medium supplemented with 0 or 3 μM ABA. Plates were incubated vertically, with seedlings placed with their root tips pointing up such that new root growth would occur along the surface of the plates in the opposite direction from the original growth. New root growth was measured after 4 d, then average growth was calculated for each genotype and treatment and expressed as a percentage of the growth on hormone-free media.

Tests of Saline or Osmotic Stress Tolerance

Seeds were surface sterilized as described above before plating on germination medium (Valvekens et al., 1988) containing 0.7% (w/v) agar. Petri plates were incubated 3 d at 4°C, then transferred to 22°C in continuous light (50–70 $\mu\text{E m}^{-2} \text{s}^{-1}$). After 2 d, germinated seedlings were transferred to germination medium supplemented with nothing, 50, or 100 mM NaCl, or 150 or 300 mM sorbitol. After 2 weeks additional growth, samples of 10 to 15 plants were harvested, blotted dry of surface moisture, and weighed. Dry weights were determined after an additional 2 to 3 d incubation in a drying oven at 65°C. Average weights per plant were calculated and expressed as a percentage of those grown on control medium.

RNA Gel-Blot Analysis

RNA was isolated from seeds and vegetative tissue by hot phenol extraction as described previously (Finkelstein, 1993). Additional seed RNA preps were based on the procedure of Vicent and Delseny (1999), modified by grinding directly in sintered glass homogenizers with extraction buffer rather than an initial grinding in liquid nitrogen with sterile quartz powder. RNA from siliques was isolated by extraction in 0.2 M Tris [tris(hydroxymethyl)-amino-methane], pH 9, 0.4 M NaCl, 25 mM EDTA, 1% (w/v) SDS, 5 mg mL^{-1} polyvinylpyrrolidone, and 0.5 mg mL^{-1} proteinase K, followed by precipitation of polysaccharides, proteins, and other contaminants by incubation on ice with 18.3 mg mL^{-1} BaCl_2 and 150 mM KCl, as described in Finkelstein et al. (1998). RNA concentrations were estimated based on absorbance at 260 and 280 nm.

Total RNA (1–20 μg per lane) was size fractionated on MOPS [3-(*N*-morpholino)-propanesulfonic acid]-formaldehyde gels (Sambrook et al., 1989), then transferred to Nytran (Schleicher & Schuell, Keene, NH) membranes using 20 \times sodium chloride/sodium phosphate/EDTA as blotting buffer. RNA was bound to the filters by UV-crosslinking (120 mJ cm^{-2} at 254 nm). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining of the filters (Herrin and Schmidt, 1988) and eventually hybridization to an rDNA probe. Transcripts from *At2S3*, *CRC*, *AtEm1*, *ABI3*, and the *RAB18* homolog were detected by hybridization to cDNA clones as described in Finkelstein (1994), labeled by random-priming to a specific activity of 10^8 cpm μg^{-1} . The *AtEm6* mRNA was

detected by hybridization to a genomic clone encompassing the entire transcribed region and 0.8-kb 5' flanking sequences. The *ABI5* probe was a PCR-amplified genomic fragment excluding most of the conserved bZIP domain. The *ABI4* probe was an *EcoRI* fragment from a cDNA clone encompassing all but the first two and final codons of the coding sequence. Hybridization conditions were (50% [w/v] formamide, 5 \times sodium chloride/sodium phosphate/EDTA, 5 \times Denhardt's, 0.1% [w/v] SDS, and 200 $\mu\text{g mL}^{-1}$ DNA) at 43°C or (7% [w/v] SDS, 0.5 M sodium-phosphate, pH 7.2, 1 mM EDTA, and 1% [w/v] bovine serum albumin) at 65°C for 16 to 24 h (Church and Gilbert, 1984) in a Hyb-Aid rotisserie oven. Filters were washed twice at 60°C in 2 \times SSC, 0.1% (w/v) SDS and once at 60°C in 0.2 \times SSC, 0.1% (w/v) SDS for 30 to 60 min. All hybridization reagents were prepared as described in Sambrook et al. (1989). Hybridization intensity was quantified by either phosphorimage analysis (Storm 640, Molecular Dynamics, Sunnyvale, CA) or densitometric analysis of autoradiograms scanned on a flatbed scanner (Vista S-8, Umax, Fremont, CA) using Photoshop to capture the images and NIH Image to calculate intensities, then normalized relative to hybridization to a radish rDNA probe (Delseny et al., 1983).

Measurement of GUS Activity

Soluble extracts of seeds were assayed fluorometrically for GUS activity using 4-methylumbelliferyl glucuronide (Rose Scientific, Canada) as substrate, as described in Jefferson et al. (1987).

GUS activity in intact plants or organs was detected histochemically by infiltration with 5-bromo-4-chloro-3-indolyl- β -glucuronic acid, as described in Jefferson et al. (1987). Plant material was cut and incubated in GUS staining solution containing 50 mM sodium phosphate, pH 7.0, 0.1% (w/v) Triton X-100, K₃/K₄ FeCN 0.5 mM, and 1 mM 5-bromo-4-chloro-3-indolyl- β -glucuronic acid at 37°C for 2 to 72 h depending on staining intensity. Tissues were cleared of chlorophyll in ethanol. Photographs of whole-mounted tissues were taken using a stereomicroscope.

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