

The Arabidopsis Abscisic Acid Response Locus *ABI4* Encodes an APETALA2 Domain Protein

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Arabidopsis abscisic acid (ABA)-insensitive *abi4* mutants have pleiotropic defects in seed development, including decreased sensitivity to ABA inhibition of germination and altered seed-specific gene expression. This phenotype is consistent with a role for *ABI4* in regulating seed responses to ABA and/or seed-specific signals. We isolated the *ABI4* gene by positional cloning and confirmed its identity by complementation analysis. The predicted protein product shows homology to a plant-specific family of transcriptional regulators characterized by a conserved DNA binding domain, the APETALA2 domain. The single mutant allele identified has a single base pair deletion, resulting in a frameshift that should disrupt the C-terminal half of the protein but leave the presumed DNA binding domain intact. Expression analyses showed that despite the seed-specific nature of the mutant phenotype, *ABI4* expression is not seed specific.

INTRODUCTION

Abscisic acid (ABA) regulates many agronomically important aspects of plant development, including synthesis of seed storage proteins and lipids (Finkelstein and Somerville, 1988; Rock and Quatrano, 1995), seed desiccation tolerance and dormancy (Black, 1983; Karssen et al., 1983; Koornneef et al., 1989), and stomatal closure (Raschke, 1979). In addition, ABA can induce tolerance of drought, salt, and cold stress (Zeevaart and Creelman, 1988; Giraudat et al., 1994). Genetic studies, especially with Arabidopsis, have identified many loci involved in ABA response (Koornneef et al., 1984; Grill et al., 1993; Finkelstein, 1994). The ABA response mutant phenotypes include defects in seed storage reserve accumulation, maturation, and dormancy and altered sensitivity to ABA or stress for control of germination inhibition, stomatal regulation, root growth, and expression of a variety of stress-induced genes (reviewed in Finkelstein and Zeevaart, 1994). Most of the mutants have relatively stage-specific defects (vegetative versus reproductive growth), and digenic analyses indicate that these loci are likely to be acting in multiple overlapping response pathways (Finkelstein and Somerville, 1990).

To date, only five mutationally identified ABA response loci have been cloned. These represent only three classes of proteins: two orthologous transcriptional regulators (Viviparous1 [Vp1] of maize [McCarty et al., 1991] and ABA-insensitive3 [ABI3] of Arabidopsis [Giraudat et al., 1992]), two

highly homologous members of the protein phosphatase 2C family (*ABI1* and *ABI2* of Arabidopsis [Leung et al., 1994, 1997; Meyer et al., 1994]), and a farnesyl transferase (enhanced response to ABA1 [ERA1] of Arabidopsis [Cutler et al., 1996]). To fully describe the molecular events in ABA signaling, we need to identify the biochemical functions of many more genes that are required for ABA response.

A single Arabidopsis *abi4* mutant was selected, on the basis of ABA-resistant germination, from an M_2 population produced by radiation mutagenesis (Finkelstein, 1994). Initial physiological and genetic analysis suggested that *ABI4* might represent a new element of the signal transduction pathway involving *ABI3*. Mutant alleles at both loci exhibited defects in seed ABA sensitivity and seed-specific gene expression but displayed normal vegetative growth. In addition, mutant alleles at both loci greatly enhanced the ABA resistance of *abi1* and *abi2* mutants but had little effect on one another. If all mutations involved in the digenic mutant analyses resulted in complete loss of function, then this would indicate that *ABI3* and *ABI4* acted in a pathway separate from *ABI1* and *ABI2*. However, interpretation of these results is complicated by the fact that the *abi1 abi3* and *abi2 abi3* digenic mutants contain a leaky *abi3* allele (Finkelstein and Somerville, 1990) and are no more resistant to ABA than are known null *abi3* mutants (Nambara et al., 1992; Ooms et al., 1993). The roles of *ABI1* and *ABI2* relative to each other and the other *ABI* genes are also complex. The recent cloning of *ABI1* and *ABI2* showed that these genes appear to encode highly similar phosphatases, leading the authors to suggest that *ABI1* and *ABI2* act redundantly (Leung et al.,

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entire 3.5-kb HindIII fragment described above, an additional 1.7 kb upstream of gene 10, and intact copies of four other genes. This apparent inconsistency suggests that the additional 1.7 kb of upstream sequence present in the cosmid negatively regulates gene 10 or that some sequences required for proper expression of gene 10 lie even farther upstream.

Having determined that the only complementing clone contained a single predicted gene that encoded a probable regulatory protein and was underexpressed in mutant tissue, we sequenced the mutant allele. We found a single base pair deletion in the coding sequence, resulting in a frameshift and early translation termination. This deletion also destroys an NlaIV site, creating an RFLP between the mutant and its progenitor line. The loss of this site was confirmed by DNA gel blot analysis (Figure 3), demonstrating that the sequence alteration detected in the polymerase chain reaction (PCR)-amplified mutant DNA was not a PCR artifact. The combination of an identified sequence mutation and the functional evidence from complementation indicate that this gene is indeed *ABI4*.

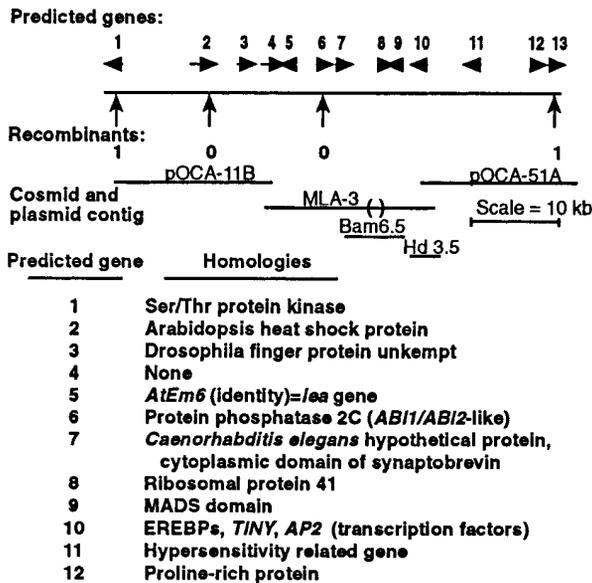


Figure 2. Genomic Organization of the Region between the Closest Flanking Recombinant Markers.

The 60 kb of the TAMU7M7 BAC between the closest flanking recombinant markers is shown. Vertical arrows indicate sites of polymorphisms scored by RFLPs or cleaved amplified polymorphic sequences. Predicted genes and their homologies are indicated, with arrowheads indicating the presumed direction of transcription. Predicted exon and intron structures of individual genes are not depicted. The cosmid and plasmid contig illustrates the extent of constructs used in complementation experiments. The parentheses by clone MLA-3 indicate a small deletion in this clone.

Table 1. Complementation of *abi4* Mutation by Transgenes

Line ^a	Intact Genes Transferred	Kanamycin Resistance ^b (%)	Germination on 5 μ M ABA ^b (%)
Col	NA ^c	NA	11 \pm 12
<i>abi4</i>	NA	NA	93 \pm 4
Bam6.5	8	100	97
MLA3-8	5,6,7,9, and 10	100	100
MLA3-9	5,6,7,9, and 10	100	86 \pm 8
Hd3.5-1a	10	100	16 \pm 13
Hd3.5-4c	10	100	13
Hd3.5-4d	10	100	10 \pm 1
Hd3.5-6b	10	100	13 \pm 1
Hd3.5-4a	10	100	70 \pm 33

^a Kanamycin and ABA sensitivity in progeny of transgenic individuals selected by growth on kanamycin was compared with that of the progenitor *abi4* line and the corresponding wild type (Col).

^b Plates were incubated for 3 or 4 days at 4°C after sowing seeds. Germination on ABA was scored after 5 days at 22°C. Kanamycin resistance was scored after 8 to 10 days. Germination percentages represent assays of seeds from individual transgenic plants. Averages are derived from assays of sibling homozygous progeny of individual transgenic lines.

^c NA, not applicable.

ABI4 Shows Homology to AP2 Domain Proteins

Analysis of the *ABI4* genomic sequence predicts a 1.3- to 1.5-kb transcript comprised of a single exon (Figure 4). Although no *ABI4* cDNAs were obtained from either silique-specific or mixed stage and tissue cDNA libraries (Giraudat et al., 1992; Newman et al., 1994), the predicted structure of *ABI4* is consistent with the sequence of cDNA clones obtained by the 3' rapid amplification of cDNA ends (RACE) technique (Frohman, 1995). The 3' ends of these clones are somewhat heterogeneous, indicating that any of several possible polyadenylation signals can be used (Figure 4A). When compared with other genes in the databases, *ABI4* shows greatest sequence homology with those encoding a class of proteins that include tobacco ethylene response element binding proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995), the Arabidopsis *TINY* gene product (Wilson et al., 1996), the Arabidopsis *CBF1* gene product (Stockinger et al., 1997), a cadmium-induced protein isolog (predicted protein from unpublished BAC sequence T01B08.3), and the AP2 protein (Jofuku et al., 1994). Although overall similarity with the closest homolog is only 30%, the conserved region is 70% similar (Figure 5). This region corresponds to the AP2 domain proposed to be involved in DNA binding and potential dimerization of this class of transcription factors. Therefore, we hypothesize that *ABI4* is also a transcription factor.

In addition to the AP2 domain located at residues 55 to 98, the *ABI4* sequence has several other characteristics consistent with function as a transcription factor: a serine/

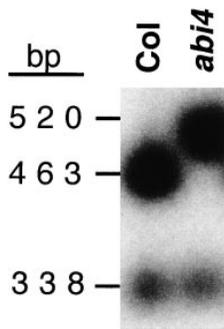


Figure 3. Gene 10 Has a Deletion in the *abi4* Mutant.

Sequence analysis of PCR products derived from the *abi4* mutant showed a 1-bp deletion at position 468 that would destroy an NlaIV site. The predicted RFLP is confirmed in this DNA gel blot comparison of wild-type and mutant genomic DNA. Numbers at left indicate the lengths of the NlaIV fragments detected by the probe.

threonine-rich (73% over 30 amino acids; residues 111 to 140) domain and a glutamine-rich (62% over 21 amino acids; residues 188 to 208) domain (Figures 4A and 4B). Less striking than the glutamine-rich domain but possibly relevant for transcriptional activation are the proline-rich (33% over 15 amino acids; residues 275 to 289) and acidic (22% over 32 amino acids; residues 295 to 326) domains. The predicted protein has two lysine/arginine-rich regions, located at residues 40 to 45 and 74 to 79 (Figure 4A). These regions might function as nuclear localization signals. Although one of these falls within a basic region of the AP2 domain that might be involved in DNA binding, this region is not conserved among AP2 domain proteins (Okamoto et al., 1997). Furthermore, many proteins have overlapping DNA binding and nuclear localization domains (Lacasse and Lefebvre, 1995). The site of the *abi4* mutation is indicated by a Δ (Figure 4). The mutant allele encodes complete presumed DNA binding, dimerization, and nuclear localization domains but would lack the glutamine-rich, proline-rich, and acidic domains, which are most likely to function in transcription activation (Mitchell and Tjian, 1989).

ABI4 Is a Member of a Gene Family

To determine whether *ABI4* is likely to have more closely related family members than those represented in the sequence databases, we used it as a probe in reduced stringency gel blot hybridizations of genomic DNA. As shown in Figure 6, there are at least 10 related sequences (Figure 6A), several of which are quite closely related (Figures 6B and 6C). When hybridized at comparable stringencies with a probe corresponding to the 3' 40% of the coding sequence, thereby excluding the AP2 domain coding region, this gene appears to have no close homologs (Figure 6D). The novel 3.2-kb Bam fragment detected by the 3' probe re-

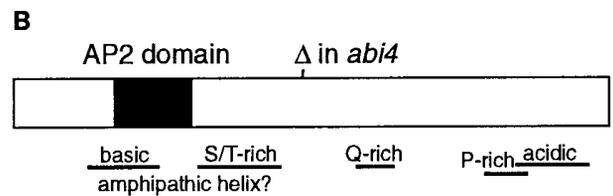
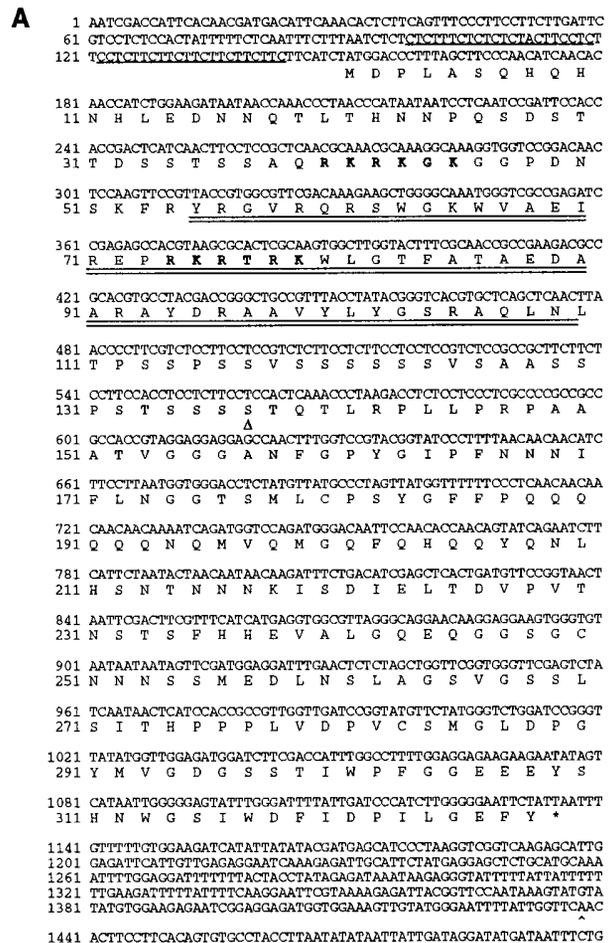


Figure 4. Sequence and Domain Structure of the *ABI4* Gene.

(A) The DNA sequence of the genomic region, including the *ABI4* coding sequence (GenBank accession number AF040959). The predicted amino acid sequence of the single open reading frame in the *ABI4* gene is shown below the DNA sequence, and the stop codon is marked with an asterisk. The location of the single base pair deletion in the *abi4* mutant is indicated by a Δ . The double-underlined region shows homology with other AP2 domain genes, as illustrated in Figure 5. Putative nuclear localization signals are in boldface. Polyadenylation sites in three independent cDNAs are indicated by carets. Sequences used as gene-specific primers for 3' RACE are underlined.

(B) Schematic diagram of the predicted *ABI4* protein, showing the locations of the AP2 domain and basic region, and the putative dimerization, serine/threonine-rich, glutamine-rich, proline-rich, and acidic domains.

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ABI4      YRGVR.QRSWG KWAIEIREPR KRT.RKWLGT FATAEDAARA YDRAAVYLYG SRAQLNLTFS
CBF1      *****NS*  ***S*V***N *K*. *I**** *Q***H**** H*V**LA*R* RS*C**FADS
Lpplzo2   *****H**  S**S***HSI LK*. *I*Q** *ES***** **E**RLMC* T**RT*FPYN
AtCdinp   *****P**  **A***D*N *AA.*V**** *D***E**L* **K**FEFR* HK*K**FPEH
Tiny      *****K*N** ***S***** *KS.*I**** *PSP*M**** H*V**LSIK* AS*I**FDDL
EREBP1    *****R*P** *FA***D*A *NGA*V**** YE*D*E**I* **K**YRMR* *K*H**FPHR
AtEBP     ***I*.K*P**  **A***D** *GV.*V**** *N***E**M* **V**KQIR* DK*K**FPDL
Ap2r1     ****TFYRRTG RWESHIWDCG K..QVY**G* D**HA***** ****TKFR*V E*DI*FNIDD
Ap2r2     ****TLHKC*R  *E*RMQFLG KKYVYLGFLF. D*EVE***** *K**IKCN*K D*V**FDPSI

EREBP-like i         fv ke             s y         i         v
Consensus YRGVR..R.wG +waAEIrd... ..R.WLgt f.t.eeAA+A YD.Aa...+G ..A..NFp..

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Figure 5. Homology between *ABI4* and Other AP2 Domain Proteins.

Shown is a comparison of the conserved regions of *ABI4* and other AP2 domain proteins. GenBank accession numbers are provided in parentheses after the gene names: *CBF1* (U77378), *TINY* (X94698), *EREBP1* (D38123), *AtEBP* (Y09942), *AP2* (U12546), *Lpplzo2* (X51767), and *AtCdinp* (Z37504). *Ap2r1* and *Ap2r2* correspond to two repeats of the AP2 domain within the AP2 protein. Amino acids identical to the *ABI4* sequence are designated by asterisks; gaps in the sequence are indicated by dots. The consensus sequence for the EREBP-like subfamily (Okamoto et al., 1997) is shown below, with invariant residues capitalized, conserved residues in lowercase, and positions that usually contain positively charged residues indicated as plus signs.

flects the fact that this fragment spans a Bam site not present in the other probe. Although it would be interesting to determine whether any of the closest homologs correspond to previously identified or novel AP2-related family members, our standard hybridization conditions should be quite selective for the *ABI4* gene.

***ABI4* Expression**

As described above, our genetic and physiological studies suggested that *ABI4* expression is likely to be seed specific in wild-type plants. To test the specificity of *ABI4* expression, we compared *ABI4* transcript levels in developing siliques versus vegetative tissue. A 1.3-kb transcript was detected in wild-type siliques but was absent or greatly reduced in *abi4* siliques and wild-type vegetative tissue (Figure 7A). Additional hybridization was observed at ~1 kb in silique RNAs. However, a more abundant smaller transcript (~850 nucleotides) was present in roots and shoots of 3-week-old plants. None of these transcripts accumulated in flowers.

The accumulation of smaller transcripts was especially surprising because the gene appears to lack introns and is therefore not a good candidate for alternative splicing. To determine whether the smaller transcripts were derived from the *ABI4* gene, we hybridized the RNA blots with a probe corresponding to ~0.5 kb of the 3' portion of the gene, excluding the conserved AP2 domain, and found that the smaller transcripts did not contain this region; however, low levels of the full-length transcript were detected in vegetative shoots (Figure 7B). In addition, we again used gene-specific primers annealing 30 nucleotides upstream of the predicted start codon in 3' RACE reactions to amplify any *ABI4* products from the vegetative tissue. No truncated products were obtained, suggesting that any such transcripts are probably not polyadenylated. Consistent with this hypothesis, the smaller

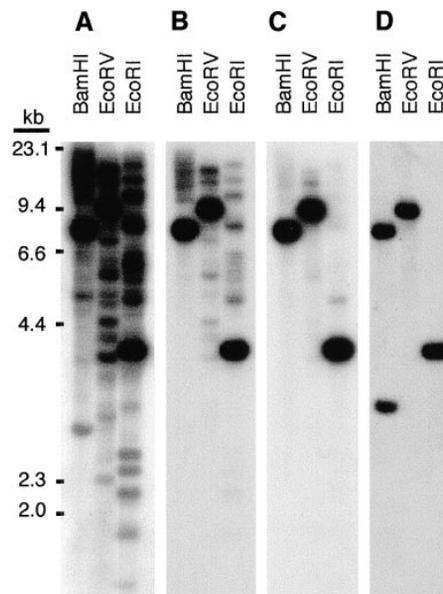


Figure 6. *ABI4* Is a Member of a Small Gene Subfamily.

Genomic Arabidopsis DNA was cleaved with BamHI, EcoRI, or EcoRV and then subjected to DNA gel blot hybridization analysis at various stringencies. In (A) to (C), the probe was a 524-bp fragment encoding residues 3 to 176, including the AP2 domain (residues 55 to 98). In (D), the probe was a 492-bp fragment encoding residues 161 to 324.

(A) A filter hybridized at 32°C below melting temperature ($T_m - 32^\circ\text{C}$) and washed at $T_m - 29^\circ\text{C}$.

(B) Same filter as shown in (A) rewash at $T_m - 24^\circ\text{C}$.

(C) Same filter as shown in (A) rewash at $T_m - 19^\circ\text{C}$.

(D) Same filter as shown in (A) hybridized at $T_m - 32^\circ\text{C}$ and washed at $T_m - 29^\circ\text{C}$.

The positions of the DNA length markers are indicated at left.

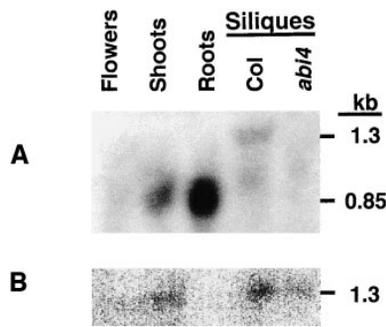


Figure 7. Expression of *ABI4*.

Shown is gel blot analysis of RNA extracted from the indicated tissues. Each lane contains 25 μ g of total RNA. Uniformity of loading and transfer was confirmed by methylene blue staining (data not shown).

(A) Hybridization with the fragment including the AP2 domain coding region.

(B) Hybridization with the fragment corresponding to the 3' portion of the gene.

Lengths of the observed transcripts are indicated at right.

transcripts were not observed in poly(A)⁺RNA from silique tissue (data not shown). However, low levels of full-length cDNAs were amplified from 11-day-old seedling RNA, again indicating that the standard *ABI4* transcript is not absolutely seed specific (Figure 8). Although our RACE PCR conditions were not designed to support quantitative comparisons, the fact that significantly less product was derived from seedling cDNA amplification suggests that the correctly sized vegetative transcript was simply below our limit of detection on the initial RNA gel blots. The identity and origin of the smaller vegetative transcript are still not clear; it might be a degradation product of the *ABI4* transcript, or it could be derived from a highly homologous family member.

DISCUSSION

The mechanisms of ABA signaling have been analyzed by biochemical and genetic approaches. Many likely signaling intermediates correlated with ABA response (e.g., ABA-activated and ABA-induced kinases and DNA binding proteins) have been identified biochemically (e.g., Guiltinan et al., 1990; Urao et al., 1993; Nelson et al., 1994; Holappa and Walker-Simmons, 1995; Knetsch et al., 1996; Li and Assmann, 1996; Soderman et al., 1996; Hong et al., 1997). However, there is not yet any genetic evidence indicating whether most of these are required for the correlated response or any other ABA-regulated processes. Genetic studies have identified signaling elements required for subsets of ABA responses, but relatively few of their biochemical identities are known. These gene products include transcription factors

(McCarty et al., 1989; Giraudat et al., 1992), protein phosphatases (Leung et al., 1994, 1997; Meyer et al., 1994), and a farnesyl transferase (Cutler et al., 1996).

The positional cloning of *ABI4* has identified a fourth protein class required for normal ABA response in seeds. Based on sequence homology, *ABI4* appears to belong to the EREBP-like class of the AP2 domain family (Okamoto et al., 1997). Similarities with this class include the presence of a single AP2 domain, containing the conserved WAAEIRD box motif, and participation in hormonal and/or stress-related signaling (Ohme-Takagi and Shinshi, 1995; Buttner and Singh, 1997; Stockinger et al., 1997). In contrast, *AP2* and *AINTEGUMENTA* each have two AP2 domains containing the WEAR/WESH motif and participate in developmental regulation of morphogenesis. There is little similarity to the other known AP2 domain family members outside of the AP2 domain. The presumed transcription activation domains of the Arabidopsis proteins AP2, CBF1, AtEBP, TINY, and the tobacco EREBPs are acidic (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995; Wilson et al., 1996; Buttner and Singh, 1997; Stockinger et al., 1997), ranging from 18 to 22% acidic over 70 to 110 amino acids. Although *ABI4* has relatively small acidic and proline-rich domains, a more obvious potential transcription activation region is glutamine rich. The unique aspects of *ABI4* are reflected in the gene-specific hybridization pattern of a probe spanning coding regions excluding that encoding the AP2 domain.

Expression analyses have shown that the full-length *ABI4* transcript is more abundant in developing siliques than in seedlings, but it is not absolutely seed specific. This is surprising because phenotypic characterization of the *abi4* mutant has suggested that *ABI4* action is necessary only during seed development (Finkelstein, 1994). The lack of correlation between expression and mutant phenotype suggests either that *ABI4* plays a minor, previously undetected role in vegetative growth or that *ABI4* action requires interaction with factors that are truly seed specific, for example, *ABI3*.



Figure 8. Full-Length cDNAs Are Amplified from Silique and Seedling RNA.

DNA gel blot hybridization of 3' RACE products from amplification of first-strand cDNA that was reverse transcribed from silique and seedling RNA. The probe was the 524-bp fragment encoding residues 3 to 176.

Alternatively, production of the ABI4 protein might be seed specific. There are many examples of post-transcriptional regulation resulting in poor correlations between transcript accumulation and mutant phenotypes for any given gene (e.g., Jack et al., 1994).

The smaller transcript present in vegetative tissue lacks the 3' end of the *ABI4* transcript and could therefore be an *ABI4* mRNA degradation product or a product of a different family member. Although the probe including the region encoding the AP2 domain hybridizes exclusively with the *ABI4* gene at high stringency, the spliced transcripts of some family members might cross-hybridize even at the high stringency of the RNA gel blot hybridizations. The *ABI4* and *TINY* genes appear to lack introns (Wilson et al., 1996), but the *AP2* gene has many introns (Jofuku et al., 1994), including several interrupting the AP2 domains. The genomic organizations of many other AP2 family members are not known because they were identified as cDNA clones (Okamuro et al., 1997).

One of our first clues regarding the identity of *ABI4* was the observation that its transcript accumulation was reduced in the mutant. Sequencing the mutant allele showed that the defect caused a frameshift within the coding sequence and that the reduced transcript levels were unlikely to result from a promoter defect. However, several alternative explanations are consistent with precedents in other systems. The frameshift and consequent early termination could result in inefficient translation, leading to decreased transcript stability and accumulation (Sullivan and Green, 1993). Furthermore, many transcription factors are autoregulatory (e.g., *DEFICIENS*; Schwarz-Sommer et al., 1992). If *ABI4* positively regulates its own expression, then the truncated protein encoded by the mutant allele might not be able to promote transcription to wild-type levels. Although the presumed DNA binding and dimerization regions of the AP2 domain are present in the mutant, the glutamine-rich, proline-rich, and acidic domains that are the regions most likely to function in transcription activation are all lost because of the frameshift.

The conserved RAYD element of the AP2 domain is also present within *ABI4*. This element is predicted to form an amphipathic α helix that could participate in protein-protein interactions that might promote DNA binding (Okamuro et al., 1997). Another member of the AP2 family, AtEBP, has been shown to interact with an octopine synthase element binding factor (OBF4). This binding factor is a member of the basic leucine zipper (bZIP) protein family (Buttner and Singh, 1997). ABA-dependent expression of cereal *Em* genes is partially mediated by interaction between the bZIP protein EmBP-1 and the conserved G-boxes present in the *Em* promoters (Gultinan et al., 1990). The Arabidopsis homolog *AtEm6* also contains a G-box within its promoter (Finkelstein, 1993; Gaubier et al., 1993), and its expression is altered in *abi4* seeds (Finkelstein, 1994), which is consistent with regulation by both bZIP and AP2 domain family members. Expression of this gene is also strongly dependent on *ABI3/Vp1* in Arabidopsis and maize, respectively (McCarty et al., 1991; Finkelstein,

1993; Parcy et al., 1994). Recently, Vp1 stimulation of transcription of the *Em* gene in HeLa nuclear extracts was shown to be dependent on the *Myc* homolog, USF, which also binds the G-box element (Razik and Quatrano, 1997). It will be interesting to learn which, if any, of these regulatory factors interact directly to control expression of *Em* and other coordinately regulated genes.

EREBP-like AP2 domain proteins have also been identified in interaction screens, using the tomato resistance gene *Pto* as "bait" (Zhou et al., 1997). The *Pto* gene encodes a serine/threonine kinase (Martin et al., 1993), so its interaction with the transcription factors is most likely to involve changes in phosphorylation states that could alter the transcription factors' cellular localization and/or activities. The predicted amino acid sequence of *ABI4* contains a serine/threonine-rich domain that could be a site of regulation by *Pto*-like kinases or serine/threonine phosphatases, such as ABI1 and ABI2. To date, no specific *in vivo* substrates for ABI1 and ABI2 have been described.

Because all of the functional domains discussed above are still hypothetical, we do not know whether loss of the glutamine-rich domain constitutes a null mutation. The phenotype of this mutant is relatively weak, producing only a five- to 10-fold decrease in sensitivity to ABA inhibition of germination and relatively subtle changes in embryonic gene expression (Finkelstein, 1994; R. Finkelstein, M. Delseny, and J. Giraudat, manuscript in preparation). In contrast, null mutations in *ABI3* result in >1000-fold decreases in ABA sensitivity for germination inhibition (Ooms et al., 1993) and complete loss of expression for several embryo-specific genes (Parcy et al., 1994). There are several possible explanations for these differences in mutant phenotype. *ABI4* may be acting downstream of *ABI3*, or at a later stage in a parallel pathway, such that the mutations have less pleiotropic effects. The *abi4* mutant may be a leaky allele if the glutamine-rich domain is not essential for its function. At least one of the *ABI4*-homologous genes detected by moderate stringency hybridizations may function redundantly and compensate for most of the lost *ABI4* activity. *ABI4* may regulate some genes positively and others negatively, as has been shown for Vp1 (Hoecker et al., 1995). The various functions might depend on different domains of the protein and/or ratios of *ABI4* protein to different interacting regulatory factors such that only some *ABI4* functions are lost in the available mutant. Having identified the *ABI4* gene, we can now test these hypotheses.

METHODS

Plant Material

The abscisic acid (ABA)-insensitive *abi4* mutant was isolated from γ -irradiated *Arabidopsis thaliana* ecotype Columbia (Col), as described previously (Finkelstein, 1994). Marker lines used for mapping

were obtained from the Arabidopsis Biological Resources Center (Ohio State University, Columbus, OH). For germination assays scoring ABA sensitivity, 20 to 100 seeds per treatment were surface sterilized in 5% hypochlorite and 0.02% Triton X-100 and then rinsed three or four times with sterile water before plating on minimal medium (Haughn and Somerville, 1986) containing 0.7% agar and ABA (mixed isomers; Sigma) at 3 or 5 μM in $15 \times 100\text{-mm}$ Petri dishes. For scoring kanamycin resistance, the medium included $0.5 \times$ Murashige and Skoog salts (Murashige and Skoog, 1962), 1% sucrose, 0.05% Mes, and 50 $\mu\text{g}/\text{mL}$ kanamycin. The dishes were incubated for 1 to 3 days at 4°C to break any residual dormancy and then transferred to 22°C in continuous light (50 to 70 $\mu\text{E m}^{-2} \text{sec}^{-1}$).

For DNA isolation, plants were grown in pots of soil (a 1:1:1 mix of vermiculite, perlite, and peat moss) supplemented with nutrient salts at 22°C in continuous light or 16-hr-light/8-hr-dark cycles; shoots and rosette leaves were harvested when the shoots started bolting. For RNA isolation from shoots, flowers, or siliques, plants were grown as described for DNA isolation. Siliques were harvested as a pooled mixture of developmental stages spanning the full period of embryogeny. For RNA isolation from roots, plants were grown hydroponically with 3 to 5 surface-sterilized seeds in 25 to 50 mL of Gamborg's B5 medium (Gamborg et al., 1968), with shaking at 70 to 80 rpm. All tissues harvested for nucleic acid extraction were weighed, frozen in liquid nitrogen, and stored frozen at -70°C until extracted.

Isolation of Recombinant Plants

The *abi4* mutant was outcrossed to marker lines CS128, CS1, and CS139 carrying the *asymmetric leaves* (*as*), *pyrimidine requiring* (*py*), and *eceriferum8* (*cer8*) mutations, respectively. Mapping populations were produced by selecting ABA-insensitive F_2 progeny and then screening the resulting F_3 families for recombinations with the markers *py*, *as*, and *cer8*, which could be scored phenotypically. To allow direct selection of recombinants between the *ABI4* and *PY* loci, an *abi4 py* recombinant was backcrossed to wild-type *Landsberg erecta* (*Ler*); ABA-insensitive F_2 progeny that were viable without a thiamine supplement were *abi4 PY* recombinants.

Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) mapping was performed with F_3 and F_4 recombinant families. Plant DNA was extracted (Jhingan, 1992), and $\sim 2 \mu\text{g}$ was digested with an appropriate enzyme to distinguish between the parental DNAs. The digested DNA was size fractionated on a 0.8% agarose gel, denatured, and transferred to Zeta Probe (Bio-Rad) membranes, as described previously (Finkelstein, 1993). Cosmid, plasmid, and phage DNA were isolated as described by Sambrook et al. (1989). DNA templates were labeled by random priming (Hodgson and Fisk, 1987), and filters were hybridized in $5 \times$ SSPE (1 \times SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), $5 \times$ Denhardt's solution (1 \times Denhardt's solution is 0.02% each of Ficoll, PVP, and BSA), 0.5% SDS, and 200 $\mu\text{g}/\text{mL}$ herring testes DNA (Sambrook et al., 1989) with 1 to 4×10^6 cpm/mL probe added. Enzymes used for scoring RFLPs between *Col* and *Ler* are given in parentheses after the marker names: *AtEm6* (SspI), g4514 (HindIII), g5054 (ClaI), m551 (ClaI), g14382 (XhoI), and m336 (ClaI).

Cleaved Amplified Polymorphic Sequence Analysis

Two cleaved amplified polymorphic sequence markers, C83 and C14k, were used to fine-map *ABI4*. Both sequences are located within the region encompassed by C1C10A6. The primers and enzymes used are available upon request. Reaction and cycling conditions were as described by Konieczny and Ausubel (1993).

Simple Sequence Length Polymorphism Analysis

The microsatellite sequence nga168 (Bell and Ecker, 1994) is polymorphic between the *Col* and *Ler* ecotypes, which are the genetic backgrounds for the *abi4* mutant and the marker lines, respectively. DNA from F_2 individuals (10 to 50 mg leaf tissue per plant) and F_3 or F_4 families was used as a template in polymerase chain reaction (PCR) amplification of the satellite sequences. PCR mixtures contained ~ 10 ng of DNA, 2.5 pmol of each primer (MapPairs from Research Genetics, Inc., Huntsville, AL), 10 mM Tris, pH 9, 50 mM KCl, 2 mM MgCl_2 , 0.01% gelatin, 0.1% Triton X-100, 200 μM deoxynucleotide triphosphates, and 0.25 units of Taq polymerase in a 10- μL reaction. Reaction products were size fractionated by electrophoresis through a 6% polyacrylamide-Tris-borate-EDTA gel (Sambrook et al., 1989).

Construction of Clones for Complementation Studies

The bacterial artificial chromosome (BAC) TAMU7M7 was used as a hybridization probe against the Arabidopsis genomic library constructed in the binary vector pOCA18hyg (Schulz et al., 1994), which is available through the Arabidopsis Biological Resources Center. We obtained three clones corresponding to the relevant portion of TAMU7M7. However, because the predicted genes 4 to 10 were not fully represented in these clones, we also constructed a mini-library of TAMU7M7 DNA in the binary cosmid vector pLCD04541. High molecular weight TAMU7M7 DNA ($\sim 2 \mu\text{g}$) was partially digested with a mixture of *Taq*I and *Taq*I methylase (2 units each; New England Biolabs, Inc., Beverly, MA) at 65°C for 30 min to generate DNA fragments ranging from 6 to 25 kb. DNA fragments (10 to 20 kb) were collected from a 0.5% low-melting-point agarose gel. The pLCD04541 binary cosmid vector (provided by C. Lister and I. Bancroft, John Innes Center, Norwich, UK) DNA ($\sim 2 \mu\text{g}$) was digested with *Cla*I and dephosphorylated. The 10- to 20-kb partially digested BAC DNA (100 ng) was ligated to the dephosphorylated vector DNA (100 ng) and transformed into *Escherichia coli* DH5 α cells. Transformants were selected on Luria-Bertani agar plates containing tetracycline and then screened by colony and DNA gel blot hybridizations (Sambrook et al., 1989) to identify clones comprising the region of interest. Several of the mini-library clones had internal deletions, so we subcloned specific fragments of TAMU7M7 into pBIN19 (Bevan, 1984) to cover individual tightly linked genes.

Transgenic Plants

abi4 plants were grown at a density of three to seven plants per 5-inch pot under 14-hr-light/10-hr-dark photoperiods to produce large leafy plants. Plants were vacuum infiltrated with an *Agrobacterium tumefaciens* culture carrying an appropriate plasmid, essentially as described by Bent et al. (1994). Seeds were harvested from individual

pots and plated on selection medium (0.5 × Murashige and Skoog salts, 1% sucrose, 50 µg/mL kanamycin, or 40 µg/mL hygromycin) to identify transgenic progeny. ABA sensitivity and antibiotic resistance were scored in the following generation.

RNA Isolation and Gel Blot Analysis

RNA was isolated from seedlings by hot phenol extraction, as described previously (Finkelstein et al., 1985). Flower and root RNA was isolated using the Plant RNeasy kit (Qiagen, Chatsworth, CA). Silique RNA was isolated by grinding siliques to a fine powder in liquid nitrogen followed by extraction for 1 hr at 37°C in 3 to 5 mL of 0.2 M Tris, pH 9, 0.4 M NaCl, 25 mM EDTA, 1% SDS, 5 mg/mL polyvinylpyrrolidone, and 0.5 mg/mL proteinase K per gram of tissue. Proteins and polysaccharides were precipitated by incubation on ice with 18.3 mg/mL BaCl₂ and 150 mM KCl. After clearing the mixture by a 10-min centrifugation at 9000g, RNA was isolated from the supernatant by LiCl precipitation. The pellets were washed in 2 M LiCl and then resuspended and reprecipitated with EtOH and NaOAc before a final resuspension in Tris-EDTA. RNA concentration was estimated based on absorbance at 260 and 280 nm.

Total RNA (25 µg per lane) was size fractionated on 1% agarose Mops-formaldehyde gels (Sambrook et al., 1989) and then transferred to Nytran (Schleicher & Schuell) or Hybond N (Amersham) membranes by using 20 × SSPE as blotting buffer. RNA was bound to the filters by UV cross-linking (120 mJ/cm² at 254 nm). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining of the filters (Herrin and Schmidt, 1988). The *ABI4* mRNA was detected by hybridization to clones corresponding to approximately the 5' and 3' halves of the coding sequence, each labeled by random priming to a specific activity of 10⁸ cpm/µg (Hodgson and Fisk, 1987). Hybridization was in 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, and 1% BSA at 65°C for 16 to 24 hr (Church and Gilbert, 1984) in a rotisserie oven (Hyb-Aid, Middlesex, UK), with probe added to 2 to 4 × 10⁶ cpm/mL. Filters were washed first in 40 mM sodium phosphate, pH 7.2, 5% SDS, and 1 mM EDTA and then in 40 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA, with a final wash in 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS for 15 to 60 min each at 65°C. Exposure times were 1 to 2 weeks.

DNA Sequence Analysis

High molecular weight BAC DNA was isolated by a modified alkaline lysis method and was fragmented by nebulization. BAC DNA (4 to 6 µg) was resuspended in 2 mL of 50 mM Tris, pH 8.0, 15 mM MgCl₂, and 25% glycerol and then transferred into a prepared nebulizer (Inhalation Plastics, Inc., Chicago, IL). DNA was nebulized for 150 sec at 30 psi at the tank outlet. Fragmented DNA sample was concentrated 4.5-fold by five or six butanol extractions and then ethanol precipitated. The DNA pellet was washed with 70% cold ethanol, dried, and then dissolved in 1 × Tris-EDTA at a final concentration of 50 µg/mL. The bulk of the DNA was fragmented into 400-bp to 3-kb pieces, as determined by gel electrophoresis. Ends of the fragmented DNA (~1.5 µg) were filled by Pfu DNA polymerase (Stratagene, La Jolla, CA). The reaction solution was loaded on a 1% low-melting-point agarose (Kodak) gel in 1 × Tris-borate-EDTA, and DNA was separated on the gel. Gel slices containing DNA fragments ranging from 500 bp to 3 kb were excised on a long-wavelength UV light (366

nm) box and then recast in another 1% low-melting-point agarose gel and run in the opposite direction to concentrate the DNA into a very thin band. The DNA band was excised and melted at 65°C for 10 min. The agarose in the solution was digested with Agarase I (New England Biolabs) at 40°C for 2 hr. The digested agarose solution was chilled on ice for 15 min and microcentrifuged at 4°C for 15 min to pellet the undigested agarose. The supernatant was transferred into another Eppendorf tube and extracted with an equal volume of phenol-chloroform and then chloroform. The extracted supernatant was mixed with 2 volumes of isopropanol, chilled on ice for 30 min, and pelleted by microcentrifugation at 4°C for 15 min. The pellet was washed with 70% cold ethanol, dried at room temperature for 15 min, dissolved in 20 µL of 1 × Tris-EDTA, and stored at 4°C for later ligation.

pBluescript II KS+ vector (Stratagene) DNA was digested with EcoRV at 37°C for 3 hr and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). The dephosphorylated vector DNA (~100 ng) was mixed with the size-selected BAC DNA (~100 ng) and ligated with T4 DNA ligase (New England Biolabs) at 16°C overnight. After ligation, the ligase was heat inhibited at 65°C for 10 min. DNA in the ligation solution was electrotransformed into *E. coli* DH5-competent cells by using a Bio-Rad gene pulser. Transformants were recovered in 1 mL of SOC (Sambrook et al., 1989) at 37°C with shaking at 300 rpm for 1 hr. Transformants in the recovery solution were mixed with an equal volume of 30% glycerol, divided into aliquots (100 µL), and stored at -80°C for later use. The frozen transformant stock was thawed briefly, diluted 50 times with Luria-Bertani liquid medium, and then plated onto selective Luria-Bertani agar plates containing 50 µg/mL ampicillin. Recombinants were identified using a blue-white screening system. White colonies were inoculated into 96-well microtiter plates (each well containing 1.3 mL of liquid Luria-Bertani medium) and cultured at 37°C with shaking at 300 rpm for 22 hr.

Plasmid DNA was isolated by using rapid extraction alkaline plasmid kits (Qiagen), dissolved in 50 µL of double-distilled water to be used as DNA templates for sequencing. DNA was sequenced on a 377 DNA sequencer (ABI Prism; Applied Biosystems, Foster City, CA). The Phred, Phrap, and Consed sequence assembly and viewing programs ([HTTP://www.genome.washington.edu/](http://www.genome.washington.edu/)) were used to remove BAC vector sequences and to assemble contigs. After large-sequence contigs were assembled and only a few gaps remained, the gaps were closed by amplifying the intervening DNA by PCR from the original BAC DNA, using primers based on adjacent sequences. The PCR products were sequenced on a long-range ABI 377 sequencer, using dye terminator chemistry, as described by the manufacturer (Perkin-Elmer).

3' Rapid Amplification of cDNA Ends

Total RNA (5 µg) was used as template for reverse transcription, using a 3' rapid amplification of cDNA ends (RACE) kit (Gibco BRL). After first-strand synthesis and RNase H treatment, cDNAs were amplified using the universal adapter primer and a gene-specific primer annealing 53 nucleotides 5' to the start of the *ABI4* open reading frame. Amplified products were size fractionated on a 1% agarose gel, blotted, and hybridized with an internal fragment of the *ABI4* gene. A nested primer, annealing 30 nucleotides 5' to the presumed initiating codon, was used to amplify cDNAs from a plug of sized DNA corresponding to the region of hybridization. The amplification reactions contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 nM each primer, 200 µM each deoxynucleotide triphosphate, and 0.05 units per µL Taq polymerase. The polymerase was

added after a 3-min incubation at 94°C for "hot start" amplification. Cycling conditions were 30 cycles of 45 sec at 94°C, 1 min at 57.5°C, and 2 min at 72°C. The cDNAs were gel purified with GeneClean (Bio-101, La Jolla, CA), according to the manufacturer's instructions, and the ends were blunted by fill-in reactions with the Klenow fragment of DNA polymerase I, followed by treatment with T4 polynucleotide kinase, as described by Sambrook et al. (1989). cDNAs were ligated into pBluescript KS+ (Stratagene) after digestion with EcoRV and dephosphorylation and then transformed into *E. coli* DH5 α . Transformants were selected on ampicillin-X-Gal-Luria-Bertani agar plates; white colonies were screened for the presence of appropriate inserts by restriction mapping of plasmid DNA.

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The Arabidopsis Abscisic Acid Response Locus *ABI4* Encodes an APETALA2 Domain Protein

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