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

Ruth R. Finkelstein, a,1 Ming Li Wang, b Tim J. Lynch, a Shashirekha Rao, a and Howard M. Goodman b

a Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, California 93106
b Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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 ABI1 [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 M2 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.,

1 To whom correspondence should be addressed. E-mail finkelst@lifesci.ucsb.edu; fax 805-893-4724.
However, the substrates for these phosphatases are not known. In addition, because the abi1 and abi2 mutant phenotypes are distinct, they are unlikely to act completely redundantly. Finally, it is not known whether the single abi4 mutation is a null or leaky allele. Given these uncertainties, many combinations of genetic interactions remain valid possibilities.

To address the molecular relationship between ABI4 and other components of the ABA signal transduction pathway(s), we used a positional cloning approach to identify the ABI4 gene. Using markers previously placed on the physical and genetic maps of chromosome 2 (Bell and Ecker, 1994; Liu et al., 1996; Zachgo et al., 1996), we localized ABI4 to a single yeast artificial chromosome (YAC). We then generated new restriction fragment length polymorphism (RFLP) markers to map the gene within a bacterial artificial chromosome (BAC) contig (Wang et al., 1997) underlying that YAC. Although we relied on functional evidence from complementation data to confirm the ABI4 gene’s identity, we initially identified candidate genes by using large-scale sequencing and sequence analysis.

The predicted ABI4 gene product shows homology to a class of transcriptional regulators that share a limited stretch of residues highly homologous to the APETALA2 (AP2) putative DNA binding domain. Thus, although they belong to different protein families, ABI4 and ABI3 also appear to be similar in that both are presumed transcriptional regulators. Expression analyses showed that unlike ABI3, ABI4 is expressed in vegetative as well as seed tissues.

RESULTS

Fine Mapping of ABI4

Our initial mapping of ABI4 localized it to the lower arm of chromosome 2, very near the simple sequence length polymorphism (SSLP) marker nga168 (Finkelstein, 1994). To generate fine-mapping populations with closely linked recombinations, we outcrossed abi4 (in the Columbia [Col] ecotype background) to lines carrying the er and py or cer8 mutations (in the Landsberg erecta [Ler] background) and screened for recombinants with these visibly scored markers. Recombinant families were subsequently scored at a series of molecular markers to identify the region of chromosome 2 that was most tightly linked to ABI4. This enabled us to fine-map ABI4 to within a single BAC, TAMU7M7 (Figure 1). This BAC was sequenced, and the genes predicted by GRAIL analysis (Lopez et al., 1994) were used to search available sequence databases. The closest recombinations in our mapping populations were nearly 60 kb apart, and the intervening DNA contained 12 predicted genes (Figure 2). These included AtEm6 (a late embryogenesis abundant gene previously shown to have altered expression in the abi4 mutant), an ABI1/ABI2 homolog, and several probable transcriptional regulators. We compared transcript levels of the ABI1/ABI2 homolog and presumed transcriptional regulators in wild-type versus abi4 siliques and found that one of these, corresponding to gene 10, showed a severely altered transcript accumulation in mutant siliques, leading us to focus our attention on this gene.

Identification of ABI4

We assembled a set of overlapping cosmid and plasmid constructs that spanned the region between the two closest recombinations (Figure 2) and then transformed these into the abi4 mutant by Agrobacterium-mediated transformation (Bent et al., 1994). Seeds from individual kanamycin-resistant T2 progeny were assayed for ABA sensitivity and kanamycin resistance to determine whether each line was hemizygous or homozygous for the transgene. Approximately 75% of the progeny of the hemizygous lines were kanamycin resistant (data not shown), which is consistent with segregation of a single transgene locus. ABA sensitivity of homozygous T3 lines is compared in Table 1. A construct transferring a 3.5-kb HindIII fragment containing only the gene whose transcript was altered in the mutant (gene 10), including 1.3 kb upstream of the coding sequence, was the only clone that complemented the mutation. The efficiency of complementation was somewhat variable, possibly reflecting positional effects of insertion sites or copy number of tandem insertions. Surprisingly, cosmid MLA-3 does not complement the abi4 mutation, even though it contains the
Cloning the Arabidopsis ABI4 Locus

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.

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/
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.

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 3'94% 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-
Cloning the Arabidopsis ABI4 Locus 1047

Cloning the Arabidopsis ABI4 Locus

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 hydrolyzed 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 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, Lplp2o2 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 (Okamura 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.

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

Genomic Arabidopsis DNA was cleaved with BamHI, EcoRV, 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 (Tm ~ 32°C) and washed at Tm ~ 29°C. (B) Same filter as shown in (A) rewarmed at Tm ~ 24°C. (C) Same filter as shown in (A) rewarmed at Tm ~ 19°C. (D) Same filter as shown in (A) hybridized at Tm ~ 32°C and washed at Tm ~ 29°C. The positions of the DNA length markers are indicated at left.
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; Sodeman 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 (Okamuro 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; Buttnner 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 EREBP are acidic (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995; Wilson et al., 1996; Buttnner 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.
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 (Itoh 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 (Guiltinan 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 placing on minimal medium (Haughn and Somerville, 1986) containing 0.7% agar and ABA (mixed isomers; Sigma) at 3 to 5 mM in 15 × 100-mm Petri dishes. For scoring kanamycin resistance, the medium included 0.5 × Murashige and Skoog salts (Murashige and Skoog, 1962), 1% sucrose, 0.05% Mes, and 50 μg/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 μE m⁻² 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 F₂ progeny and then screening the resulting F₂ 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 F₂ progeny that were viable without a thiamine supplement were abai4 PY recombinants.

Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) mapping was performed with F₂ and F₄ recombinant families. Plant DNA was extracted (Higian, 1992), and ~2 μg 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 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% each of Ficoll, PVP, and BSA), 0.5% SDS, and 200 μg/mL herring testes DNA (Sambrook et al., 1989) with 1 to 4 × 10⁶ cpn/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 (Clal), m551 (Clal), g14382 (Xhol), and m336 (Clal).

Cleaved Amplified Polymorphic Sequence Analysis

Two cleaved amplified polymorphic sequence markers, CB3 and C14k, were used to fine-map ABI4. Both sequences are located within the region encompassed by CIC10A6. 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 F₂ individuals (10 to 50 mg leaf tissue per plant) and F₃ or F₄ 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 9.5, 50 mM KCl, 2 mM MgCl₂, 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 (~2 μg) was partially digested with a mixture of TaqI and TaqI 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, J ohn Innes Center, Norwich, UK) DNA (~2 μg) was digested with Clal 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 DH5α 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 RNAeasy 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 10 vol of isopropanol, and 0.5 mg/mL proteinase K per gram of tissue. The supernatant was transferred into another Eppendorf tube and extracted with an equal volume of phenol-chloroform and then chloroform. The collected supernatant was mixed with 2 vol 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 ligations.

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 inactivated 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/) 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).

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 vol 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 ligations.

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 50 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

Cloning the Arabidopsis ABI4 Locus 1051
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 DH5α. 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.

ACKNOWLEDGMENTS

We thank Dr. Eva Soderman for critical review of the manuscript and subcloning of the RsaI-EcoRI fragment used as a gene-specific probe; Maureen Dolan, Steven Belmonte, and Ulandt Kim for assistance with sequencing and preparation of exon-specific probes for use on RNA gel blots; and Drs. Clare Lister and Ian Bancroft, who probe; Maureen Dolan, Steven Belmonte, and Ulandt Kim for assistance with sequencing and preparation of exon-specific probes for use on RNA gel blots; and Drs. Clare Lister and Ian Bancroft, who provided the pLCD04541 cosmid binary vector and information on subcloning. This work was supported by grants to R.R.F. from the National Science Foundation (No. DCB-9105241) and the U.S. Department of Agriculture (No. 95-37304-2217) and by financial support from Hoechst AG to H.M.G.

Received January 14, 1998; accepted February 17, 1998.

REFERENCES


Cloning the Arabidopsis ABI4 Locus


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

Ruth R. Finkelstein, Ming Li Wang, Tim J. Lynch, Shashirekha Rao and Howard M. Goodman

Plant Cell 1998;10;1043-1054
DOI 10.1105/tpc.10.6.1043

This information is current as of June 29, 2017

References
This article cites 59 articles, 34 of which can be accessed free at:
/content/10/6/1043.full.html#ref-list-1

Permissions

eTOCs
Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain

CiteTrack Alerts
Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain

Subscription Information
Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY