Isolation of the Arabidopsis *ABI3* Gene by Positional Cloning

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Arabidopsis *abi3* mutants are altered in various aspects of seed development and germination that reflect a decreased responsiveness to the hormone abscisic acid. The *ABI3* gene has been isolated by positional cloning. A detailed restriction fragment length polymorphism (RFLP) map of the *abi3* region was constructed. An RFLP marker closely linked to the *abi3* locus was identified, and by analyzing an overlapping set of cosmid clones containing this marker, the *abi3* locus was localized within a 35-kb region. An 11-kb subfragment was then shown to complement the mutant phenotype in transgenic plants, thereby further delimiting the position of the locus. A candidate *ABI3* gene was identified within this fragment as being expressed in developing fruits. The primary structure of the encoded protein was deduced from sequence analysis of a corresponding cDNA clone. In the most severe *abi3*-4 allele, the size of this predicted protein was reduced by 40% due to the presence of a point mutation that introduced a premature stop codon. The predicted *ABI3* protein displays discrete regions of high similarity to the maize *viviparous-1* protein.

INTRODUCTION

The phytohormone abscisic acid plays an essential regulatory role for a variety of physiological processes, including embryo development, seed dormancy, transpiration, and adaptation to environmental stresses (Zeevaart and Creelman, 1988; Davies and Jones, 1991). The molecular mechanisms of abscisic acid action, in particular the underlying signal perception and transduction pathways, remain largely unknown. Saturable abscisic acid binding sites have been described (Hocking et al., 1978; Hornberg and Weiler, 1984), but the corresponding proteins have not been characterized. In stomatal guard cells, abscisic acid appears to play a direct role in regulating the rapid alteration of ion fluxes, whereas the remaining abscisic acid–mediated responses involving long-term changes appear to act at the level of gene expression. Several abscisic acid–regulated genes have been isolated by differential hybridization (reviewed by Skriver and Mundy, 1990), and candidate *cis*-acting elements (Marcotte et al., 1989; Mundy et al., 1990) and the corresponding transcription factors (Guiltnan et al., 1990; Oeda et al., 1991) have been characterized.

Mutational analysis represents an alternative approach for the identification of elements of the abscisic acid response pathway(s). Mutants with a reduced sensitivity to abscisic acid have been isolated in a variety of species (reviewed by Koornneef, 1986), the best characterized being those in maize and Arabidopsis. Mutations at the maize *viviparous-1* (*VP1*) locus have pleiotropic effects confined to seed development (Robertson, 1955; Robichaud et al., 1980); the *VP1* gene has been cloned by transposon tagging and has been shown to encode a potential transcription activator (McCarty et al., 1991).

In Arabidopsis, three genetically distinct "abscisic acid-insensitive" loci (*ABI1*, *ABI2*, and *ABI3*) have been identified (Koornneef et al., 1984). These mutants were selected based on the ability of seeds to germinate in the presence of inhibitory concentrations of abscisic acid. Mutations at all three loci result in reduced seed dormancy and up to a 10-fold decrease in sensitivity of seed germination and seedling growth to inhibitory concentrations of abscisic acid. The *abi3* mutations have further been shown to affect several additional aspects of seed development, including accumulation of storage proteins and lipids, chlorophyll breakdown, and desiccation tolerance (Koornneef et al., 1984, 1989; Finkelstein and Somerville, 1990; Nambara et al., 1992). These processes are apparently unaffected in *abi1* and *abi2* mutants. Conversely, *abi1* and *abi2*, but not *abi3*, plants transpire excessively, are prone to wilting, and display a reduced accumulation of proline in response to exogenous abscisic acid (Koornneef et al., 1984; Finkelstein and Somerville, 1990). Based on these
phenotypic differences, it has been suggested that the ABI3 gene product acts largely at the level of seed development, whereas ABI1 and ABI2 act at the vegetative level (Koornneef et al., 1984; Finkelstein and Somerville, 1990).

Two lines of evidence suggest that the abi mutations affect the response to abscisic acid, as opposed to either the synthesis or metabolism of abscisic acid. First, in contrast to the Arabidopsis abscisic acid–deficient mutants (aba), which have greatly reduced endogenous levels of abscisic acid, the developing seeds of the abi mutants display slightly higher levels of abscisic acid relative to wild type (Koornneef et al., 1984). Second, the mutant phenotypes are unaffected by the exogenous application of abscisic acid (Koornneef et al., 1984). The pleiotropy conferred by the abi mutations, coupled with the response phenotype, is consistent with the possibility that the ABI loci encode elements of abscisic acid signal perception/transduction cascades. Cloning of the ABI genes will provide valuable tools for the molecular dissection of these pathways, as well as their interrelationship.

Arabidopsis presents several unique features that facilitate the cloning of genes for which only a mutant phenotype and not the protein of the gene is known (reviewed by Meyerowitz, 1989). Identification of such genes ultimately relies on functional complementation of the mutation in transgenic plants. Despite the moderate size of the Arabidopsis genome (100 Mb), it is currently necessary to limit the number of clones to be tested by first delineating a small genomic region that contains the locus of interest. A strategy that proved efficient for this purpose in other organisms is that of positional cloning (Wallace et al., 1990), where the gene is isolated based on its chromosomal map location. The first step involved is to identify DNA probes residing in the vicinity of the locus of interest; such linked molecular markers can be found in the available restriction fragment length polymorphism (RFLP) linkage maps of the Arabidopsis genome (Chang et al., 1988; Nam et al., 1989). It is then necessary to bridge the intervening gap between the nearest RFLP marker and the target locus. This can be achieved by undertaking a chromosome walk, i.e., isolating sets of overlapping genomic clones to successively approach the locus, or, alternatively, by taking advantage of a physical map, which is a collection of pre-ordered overlapping clones. A physical map of the Arabidopsis genome that is correlated to the RFLP map is being constructed; it presently consists of 750 sets of overlapping cosmids clones and covers approximately 95% of the Arabidopsis nuclear genome (Hauge et al., 1991).

We have undertaken the cloning of the Arabidopsis ABI loci and report here the isolation of the ABI3 gene. By using the integrated physical/RFLP map, the locus was first localized within a single genomic cosmid clone. A subfragment of this clone was then shown to be able to complement the mutant phenotype, thereby further delimiting the location of the locus. Finally, cDNA library screening and sequence analysis of the most severe mutant allele, abi3-4, were used to identify the ABI3 gene.

RESULTS

Identification of an RFLP Marker Closely Linked to ABI3

As a first step in the cloning of ABI3, a search was conducted for tightly linked molecular markers using the available Arabidopsis RFLP maps. Populations segregating for the abi3-1 mutation were used for RFLP segregation analysis to more precisely localize the markers with respect to the ABI3 locus. To minimize the number of progeny needed for the analysis, and at the same time ensure an enhanced resolution in the vicinity of the ABI3 locus, recombinants were selected between the ABI3 locus and genetic markers flanking the locus.

The ABI3 locus had been assigned by Koornneef (1987) to map position 23.5 cM on chromosome 3, and is bracketed by HY2 (long hypocotyl-2) at 0.0 cM and GL1 (glabrous-1) at 40.9 cM, as shown in Figure 1A. Two populations of recombinants were isolated, one in the HY2-ABI3 interval (population I, plants named I-#) and the other in the ABI3-GL1 interval (population II, plants named II-#). As a consequence of construction, all of these plants were homozygous Landsberg (mutant background) at the ABI3 locus and heterozygous Landsberg/Columbia at the flanking loci (see Methods).

The RFLP map of the Arabidopsis genome constructed by Nam et al. (1989) includes the HY2 and GL1 genetic markers on chromosome 3. These were used as reference points to tentatively align the genetic and RFLP maps and to infer the approximate position of the ABI3 locus with respect to the RFLP markers. The mapping data predicts that the ABI3 locus is located between marker cosmid 6220 and the cluster of markers cosmid 4711 and λba433 and cosmid 17287 (Figure 1A). The segregation of the corresponding RFLPs was analyzed using recombinants of the above populations I and II. Marker 6220 mapped on the HY2 proximal side of ABI3. Of 31 plants tested from population I, 13 had a recombination break point in the ABI3-6220 interval. On the other hand, out of 30 plants from population II analyzed, 1, 3, and 7 plants had a recombination break point in the 4711-, 17287- and λba433-ABI3 intervals, respectively. These markers thus mapped on the GL1 proximal side of ABI3 in the order GL1-λba433-17287-4711-ABI3, as shown in Figure 1B. These data indicated that of all the markers tested, 4711 was the closest to the ABI3 locus, as it detected only one recombinant (plant II-49).

As shown in Figure 2, probe 4711 detected four Landsberg-specific (1L, 2L, 4L, 6L) and four Columbia-specific (3C, 5C, 7C, 8C) polymorphic bands in HindIII digests. This pattern indicated that several distinct polymorphic HindIII restriction sites were detected by this probe. These sites are genetically linked, as all of the polymorphic bands cosegregated in the F2 progeny analyzed by Nam et al. (1989) and were used as a set by the authors to define the 4711 marker (H. M. Goodman, unpublished results). Furthermore, these polymorphic sites appeared to be located within the 4711 insert itself, as the
Cloning the Arabidopsis AB13 Locus

A

<table>
<thead>
<tr>
<th>40.9</th>
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<tr>
<td>gl1</td>
<td>abi3</td>
<td>hy2</td>
</tr>
</tbody>
</table>

B

\[ \frac{30}{7/30} = 4.28 \text{ cM} \]

C

\[ \frac{7/30}{1/69} = 0.25 \text{ cM} \]

Figure 1. Schematic Summary of RFLP Analysis Data.

(A) Alignment of the genetic (top) and RFLP (bottom) maps of the relevant portion of chromosome 3. Adapted from Koornneef (1987) and Nam et al. (1989).

(B) Refined RFLP map in the vicinity of the abi3 locus. For each RFLP marker, the number of plants from populations I (6220) or II (IbaI 433, 17267, and 4711) analyzed is indicated, as well as the number of recombination break points identified in the corresponding abi3-marker interval.

(C) Location of the abi3 locus in the set of overlapping cosmid clones. The set was assembled based on the fingerprinting of individual random clones (Hauge et al., 1991). Clones are depicted by solid lines, the length of which is proportional to the number of bands in its fingerprint; only a few members of the set are shown here. For RFLP markers 4711-7 and 4711-8, the number of plants from populations I (4711-8) or II (4711-7) analyzed is indicated, as well as the number of recombination break points identified in the corresponding abi3-marker interval.

HindIII digest of clone 4711 (isolated from a Columbia genomic library) contained bands that comigrated with the 3C, 5C, 7C, and 8C polymorphic bands, as shown in Figure 2. The 4711 HindIII fragments comigrating with bands 7C and 8C were subcloned to yield clones 4711-7 and 4711-8, respectively. When used as a hybridization probe, each of these subclones detected a unique RFLP site and hybridized to the expected polymorphic Columbia band (Figure 2), thus confirming the location of these two sites within the insert of clone 4711.

Upon hybridization to DNA from plant II-49, clone 4711 detected only one of the Columbia-specific bands (7C; Figure 2). This particular plant therefore contained a recombination break point located within the region defined by 4711, as evidenced by hybridization with subclones 4711-7 and 4711-8 (Figure 2). Plant II-49 was heterozygous Landsberg/Columbia at the level of the 4711-7 polymorphic site, indicating that this marker detected a recombinant in population II and thus mapped on the GL7 proximal side of AB13. To better estimate the genetic distance between RFLP 4711-7 and the AB13 locus, a total of 69 (the above 30 plus 39 others) plants from population II were analyzed. No additional recombinant, other than plant II-49, was identified. Assuming that the recombination break points in this population were randomly distributed in the selected AB3-GL7 interval, the 4711-7 RFLP site was predicted to be within 0.25 cM \((40.9 \text{ cM} - 23.5 \text{ cM}) \times 1/69\) from the abi3-1 mutation.

Analysis of an Overlapping Cosmid Set Containing Clone 4711

Marker 4711 is a cosmid clone that has been incorporated into the physical map of the Arabidopsis genome constructed by Hauge et al. (1991), and is located near one end of a small set of overlapping cosmid clones shown in Figure 1C. To orient this set of overlapping clones with respect to the genetic map, clone 6974 (located near the other end of the set) was used as an RFLP probe. Clone 6974 detected no recombinant in population II, but did detect two recombinants (plants I-34 and I-147) in population I; thus, this marker mapped on the HY2 proximal side of AB13. The set of overlapping clones appeared to encompass the AB13 locus.

To further refine the position of the locus, the internal clone 3669 was used as an RFLP probe; it detected the same two recombinants as 6974. Plants from population I (68 in total) were then probed with 4711. All of the plants, with the exception of I-34 and I-147, were homozygous Landsberg. For these two plants, the 4711 hybridization patterns were identical and contained the two Columbia-specific bands 5C and 8C (Figure 2). Hybridization with 4711 subclones demonstrated that both plants were heterozygous Landsberg/Columbia at the 4711-8 RFLP site and homozygous Landsberg/Landsberg at the 4711-7 RFLP site. The 4711-8 RFLP site was thus located on the HY2 proximal side of AB13, at \(-0.70 \text{ cM}\) distance \([(23.5 \text{ cM} - 0.0 \text{ cM}) \times 2/68]\) from the abi3-1 mutation.

The above data showed that, among the various HindIII polymorphic sites detected by probe 4711, the 4711-7 and 4711-8 RFLP sites mapped on either side of the abi3-1 mutation. This mutation therefore had to be located within the region delimited by the insert of clone 4711. The positions of fragments 4711-7 and 4711-8 on the restriction map of clone 4711, shown in
Figure 2. Autoradiograms of DNA Gel Blots Used for the Detection of RFLPs.

Total genomic DNA was extracted from wild-type Columbia (Col) ecotype, wild-type Landsberg erecta (Lan) ecotype, abi3-1/abi3-1 mutant line (abi3), and recombinant plants II-49, I-34, and I-147. These genomic DNAs and DNA from cosmid 4711 were digested by HindIII and submitted to gel blot hybridizations using the various probes indicated. In each case, the Landsberg- (L) and Columbia- (C) specific polymorphic bands detected are indicated.

Figure 3, indicated that the entire ABI3 gene was likely to be contained within this clone.

Functional Complementation

Three overlapping restriction fragments of the 4711 Columbia wild-type genomic clone were tested for their ability to complement the abi3-1 mutation (i.e., restore wild-type sensitivity to exogenous abscisic acid at the germination stage). The fragments 4711-K, 4711-E, and 4711-S (see Figure 3) were cloned into the disarmed T-DNA vector pGSFR161, which carries a kanamycin resistance marker for plant selection (Valvekens et al., 1988). Agrobacterium strains harboring these constructs were used to transform root explants of the Landsberg abi3-1 mutant. Several kanamycin-resistant regenerants were obtained; however, because none of these transformants produced seeds, it was impossible to assay their abi3 phenotype (which is confined to the seed and seedling stages). By contrast, parallel experiments using root explants of the wild-type C24 line yielded numerous fertile transformants. Therefore, the following strategy was adopted to test for functional complementation.

For each construct, the C24 transformed lines were assayed for ploidy. Several independent diploid lines, which segregated approximately 3:1 for kanamycin resistance and therefore should contain transferred DNA at a single locus, were retained for further study. Each line was crossed to the abi3-1 mutant; kanamycin-resistant F1 plants (heterozygous abi3-1 [Landsberg]/+ [C24] at the ABI3 locus and hemizygous for the T-DNA insertion) were selected and allowed to self. The segregation of the abi3-1 phenotype was analyzed in the resultant F2 progeny. If no functional complementation took place, all of the F2 seeds with the abi3-1/abi3-1 genotype (1/4 of the F2 population) were expected to be abscisic acid resistant. If the construct did complement the mutation, only those F2 seeds with the abi3-1/abi3-1 genotype and that did not inherit the T-DNA would be abscisic acid resistant.

As shown in Table 1, fragments 4711-K and 4711-S did not complement the mutation; approximately 25% of the F2 seeds of all progeny analyzed were abscisic acid resistant and a large portion of these were also kanamycin resistant. By contrast, complementation was observed with fragment 4711-E in seven...
Table 1. Segregation of Abscisic Acid and Kanamycin Resistance in F2 Populations

<table>
<thead>
<tr>
<th>Construct</th>
<th>3 μM Abscisic Acid</th>
<th>50 mg/L Kanamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Random(^a)</td>
<td>Abscisic Acid Resistant</td>
</tr>
<tr>
<td></td>
<td>res/test</td>
<td>%</td>
</tr>
<tr>
<td>4711-K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>83/302(^b)</td>
<td>27.5</td>
<td>25/36</td>
</tr>
<tr>
<td>27/104(^b)</td>
<td>26.0</td>
<td>16/24</td>
</tr>
<tr>
<td>20/104(^b)</td>
<td>19.2</td>
<td>15/20</td>
</tr>
<tr>
<td>29/105(^b)</td>
<td>21.9</td>
<td>13/21</td>
</tr>
<tr>
<td>26/100(^b)</td>
<td>23.3</td>
<td>15/24</td>
</tr>
<tr>
<td>28/100(^b)</td>
<td>27.2</td>
<td>15/26</td>
</tr>
<tr>
<td>42/156(^b)</td>
<td>26.9</td>
<td>n.t.</td>
</tr>
<tr>
<td>4711-S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49/208(^b)</td>
<td>23.6</td>
<td>33/49</td>
</tr>
<tr>
<td>55/208(^b)</td>
<td>26.4</td>
<td>26/32</td>
</tr>
<tr>
<td>48/208(^b)</td>
<td>23.1</td>
<td>25/27</td>
</tr>
<tr>
<td>51/208(^b)</td>
<td>28.4</td>
<td>42/59</td>
</tr>
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<td>53/208(^b)</td>
<td>24.5</td>
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</tr>
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<td>21/26</td>
</tr>
<tr>
<td>4711-E</td>
<td></td>
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<tr>
<td>41/582(^c,d)</td>
<td>7.0</td>
<td>0/10</td>
</tr>
<tr>
<td>16/608(^c)</td>
<td>7.7</td>
<td>0/14</td>
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</tr>
<tr>
<td>16/106</td>
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<tr>
<td>27/106(^c)</td>
<td>25.5</td>
<td>18/26</td>
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\(^a\)For each construct, the data for the F2 progeny derived from several independent transgenic parents are shown. Random F2s were assayed for abscisic acid resistance; random as well as selected abscisic acid-resistant F2s were assayed for kanamycin resistance.

\(^b,c,d\)For each F2 population, the fraction (9 of tested seeds displaying abscisic acid resistance was analyzed by $\chi^2$ tests of goodness-of-fit: \(r^2\) for resistance tested.

\(^f\) = 1/4, \(p > .1\), \(^{cf}\) = 1/4, \(p < .001\), \(^{df}\) = 1/16, \(p > .05\).

\(^{n.t.}\), not tested.

of the nine progeny analyzed, where clearly less than 25% of the F2 seeds were abscisic acid resistant. In four of the progeny, the abscisic acid-resistant seeds constituted roughly 1/16 of the F2 population, as would be expected if in the parental C24 plant the T-DNA integration site was genetically unlinked to the ABI3 locus. When the abscisic acid-resistant F2 plants derived from the above seven progeny were retested on kanamycin, no resistance was observed in five cases and only 12 to 13% were resistant in the other two.

Additional evidence that fragment E was capable of functionally complementing the abi3-1 mutation was obtained by analyzing individual abscisic acid-sensitive F2 plants. Random F2 seeds from a single population were sown on plates containing 3-μM abscisic acid and, after a week, abscisic acid-sensitive seeds were transferred to nonselective medium to allow germination and growth. DNA was extracted from random individual plants, digested with HindIII, and characterized by DNA gel blot analysis using a cDNA clone (see below) corresponding to a subregion of fragment 4711-E (Figure 3) as the probe. This probe detected distinct bands characteristic of (1) wild-type C24, (2) abi3-1 Landsberg mutant, and (3) the T-DNA insertion. Therefore, the genotype at this particular locus, as well as the presence or absence of the T-DNA, could be determined for each individual plant. As shown in Figure 4, of the 19 abscisic acid-sensitive plants analyzed, four displayed only the abi3-1 and T-DNA bands; therefore, these individuals were homozygous abi3-1/abi3-1 at the ABI3 locus, and their abscisic acid-sensitive phenotype resulted from functional complementation and not from inheritance of the wild-type C24 allele.

Identification of the ABI3 Gene within Fragment 4711-E

Complementation experiments indicated that the ABI3 gene is located within fragment 4711-E. Because abi3 mutations have pleiotropic effects on seed development, the ABI3 gene is expected to be expressed in developing fruits. Fragment 4711-E was thus used as a probe for RNA gel blot analysis. As shown in Figure 5, this probe detected a 3-kb mRNA in green (unripe)}
Individual F2 plants

T-DNA
abi3

C24

Figure 4. Complementation of the abi3-1 Mutation in Individual F2 Plants.

Autoradiogram of a DNA gel genomic blot probed with the 2.9-kb cDNA pcabi3-4F. Abscisic acid-sensitive (wild-type phenotype) plants were selected in the F2 progeny derived from a cross between the abi3-1 mutant and a transgenic C24 plant containing the 4711-E construct. Genomic DNAs from the abi3-1 mutant (abi3), wild-type untransformed C24 line (C24), transgenic C24 parent (C24 T), and 19 random individual abscisic acid-sensitive F2 plants were digested with HindIII and submitted to gel blot analysis. The distinct bands originating from the T-DNA insertion (T-DNA), Landsberg abi3-7 allele (abi3), and C24 wild-type allele (C24), respectively, are indicated. The lanes corresponding to individuals in which the wild-type phenotype results from functional complementation are indicated by asterisks.

siliques, for which an apparently near full-length (2.9 kb) cDNA clone was then isolated (see Methods). The corresponding gene was mapped by determining and aligning the complete nucleotide sequences of the cDNA and of the 4711-E fragment. This gene was located in the only region of fragment 4711-E that displayed open reading frames longer than 300 bp (data not shown). Furthermore, it was contained within the complementing fragment 4711-E, but was truncated and absent in the noncomplementing fragments 4711-K and 4711-S, respectively (Figure 3).

When used as an RFLP probe, the 2.9 kb cDNA clone detected a HindIII polymorphic site which cosegregated with the abi3 locus in all recombinant plants from both populations I and II (Figure 2). Several abi3 mutant alleles were tested by DNA and RNA gel blot analyses using fragment 4711-E as the probe; this study did not reveal any genomic rearrangement or significant modification of the size and abundance of the siliques 3-kb transcript. The most severe abi3-4 mutant allele, which has recently been isolated by K. Leon and M. Koornneef (personal communication), was thus compared to the wild-type Landsberg line by sequence analysis. In this mutant, the genomic region corresponding to cDNA clone pcabi3-4F was amplified by polymerase chain reaction from total DNA of either the wild-type Landsberg or abi3-4 mutant line and submitted to sequence analysis. In the abi3-4 allele, nucleotide C 1654 is replaced by a T, which converts the CAA codon encoding Gln 417 into a TAA premature stop codon.

The long open reading frame predicts that ABI3 encodes a protein of 720 amino acids, with a 79500-D molecular weight and an isoelectric point of 5.13. Inspection of the primary structure, shown in Figure 7, reveals that this polypeptide chain is composed of several domains with distinct characteristics, as summarized in Figure 6B. From residue 1 to 78 an acidic domain, from 79 to 123 a neutral domain highly enriched (51%) in hydroxylated residues, from 124 to 254 an acidic domain, from 255 to 310 a basic domain, from 311 to 438 an acidic domain rich in Pro (25%) and Asn + Gln (22%) residues, from 439 to 476 a basic stretch that contains the putative nuclear

Sequence Analysis of the ABI3 Gene

The nucleotide sequence of the cDNA clone pcabi3-4F (EMBL accession number X68141) contains 2868 bp, including a 3' poly(A) tail, a value in close agreement to the size of the mRNA as determined by RNA gel blot analysis (3 kb). Comparison with the sequence of the genomic fragment 4711-E revealed the presence of five introns (200, 145, 133, 117, and 110 bp long, respectively), the positions of which are shown in Figure 6A. The cDNA insert contains a large open reading frame starting at nucleotide 406 and ending at nucleotide 2565. Several putative ATG initiation codons are found 5' to this long open reading frame; however, each of them is followed by an in-frame stop codon. Also, the sequences surrounding these ATGs do not match the plant initiation codon consensus sequence (Lütcke et al., 1987), whereas the context of the proposed ABI3 translation start site (AACAUGUGA) fits that consensus (AACAUGGCC) better.

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These convergent lines of evidence indicated that the 2.9-kb cDNA (pcabi3-4F) is actually derived from the ABI3 transcript.

Figure 5. Autoradiogram of an RNA Gel Blot Probed with Fragment 4711-E.

The blot contains 0.5 μg of poly(A)+ RNA isolated from Arabidopsis stems, green siliques (all developmental stages pooled), and leaves.
targeting signal RKKR (Chelsky et al., 1989), from 477 to 556 (A)

Figure 6. Schematic Structures of the ABI3 Gene and Protein. (A) Organization of the genomic region corresponding to cDNA clone pcabi3-4F. The exons are indicated by heavy lines. Positions of the ATG and TGA codons delimiting the large open reading frame are indicated. (B) Structural domains of the predicted amino acid sequence of the ABI3 protein. The upper line indicates the borders of the different coding exons. The acidic and basic domains are depicted by hatched and stippled boxes, respectively. Abundant amino acid residues are indicated underneath each box. Domains with high sequence similarity to the maize VP1 protein are indicated by asterisks.

A

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1 kb

B

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<th>N-Q</th>
<th>S+T</th>
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Figure 7. Alignment of the Predicted Amino Acid Sequences of the Arabidopsis ABI3 and maize VP1 proteins. ABI3 protein sequence, top lines; VP1 protein sequence, bottom lines. The alignment was obtained with the GAP program, using a gap weight of 0.3 and a gap length weight of 0.1.

DISCUSSION

Several lines of evidence support the identification of the ABI3 gene. This genomic region cosegregates with the abi3 locus in the recombinant populations I and II, which provide resolutions of 0.35 cm and 0.25 cm in the ABI3/HY2 and ABI3/GL7 intervals, respectively. The identified transcription unit is intact in the complementing fragment 4711-E, but not in the noncomplementing fragments 4711-K and 4711-S. The complementing fragment 4711-E does not appear to contain any
additional genes, as judged from nucleotide sequence analysis and RNA gel blot hybridization. Finally, the identified gene is expressed in developing siliques, as would be expected for the ABI3 gene based on the mutant phenotypes, and carries a mutation that leads to a truncation of the encoded protein in the most severe abi3-4 mutant allele.

This study demonstrates the feasibility of isolating an Arabidopsis locus by positional cloning. This species has gained increasing popularity as a model system for the study of plant biology, mainly because it should facilitate the isolation of genes that have been identified by mutational analysis. Several Arabidopsis loci have already been cloned, either by taking advantage of available T-DNA tagged alleles (Herman and Marks, 1989; Koncz et al., 1990; Yanofsky et al., 1990) or by genomic subtraction (Sun et al., 1992). However, these two strategies require the availability of appropriate alleles, which can conceivably be difficult to obtain in certain cases. In contrast, positional cloning can be applied to any mutant, regardless of the mutagenesis method by which it was obtained; thus, it provides a more generally useful alternative.

Although the present study benefited from an exceptional situation (target gene within the starting clone), it illustrates several points of broader relevance. This work demonstrates that, as previously discussed by Nam et al. (1989), the nearest RFLP marker to a given locus cannot be identified with confidence simply by aligning the genetic and RFLP maps of the Arabidopsis genome. Indeed, this procedure positions the ABI3 locus several centiMorgans away from the 4711 marker, which in fact contains the ABI3 gene. As more loci are cloned, a reliable integrated genetic/RFLP map will progressively emerge; until then, the effort made here to refine the RFLP map in the vicinity of the target locus appears necessary. This fine mapping indicated that the 4711-7 and 4711-8 RFLP markers are separated by 0.95 cM and ~30 kb, whereas an average value of ~200 kb/cM is anticipated for the Arabidopsis genome (Chang et al., 1988; Nam et al., 1989). As observed in other organisms, the kilobase to centiMorgan correspondence is thus likely to vary significantly from one region of the genome to another. The availability of a physical map covering the region of interest clearly obviates the need for labor-intensive chromosome walking. In Arabidopsis, sets of overlapping cosmid clones have the further advantage, as compared to yeast artificial chromosomes (Hwang et al., 1991), that each cosmid clone can be directly used as an RFLP probe.

The characteristics of the existing abi3 mutant alleles support the hypothesis that the ABI3 protein participates in an abscisic acid perception/transduction cascade. Data base searching did not reveal any significant homology to the various protein families known to be involved in such functions in the case of other chemical signals. The ABI3 protein contains a putative nuclear targeting sequence, and several regions of this polypeptide present features previously described in activation domains of transcription regulators: acidic, Gln rich, Pro rich, or Ser + Thr rich, respectively (reviewed by Mitchell and Tjian, 1989). These observations thus raise the hypothesis that the ABI3 protein participates in abscisic acid–regulated gene expression during seed development.

Sequence similarity was found between the ABI3 protein and the maize VP1 protein. Embryos homozygous for the maize vp1 mutation exhibit a reduced sensitivity to abscisic acid resulting in precocious germination, whereas the synthesis and metabolism of abscisic acid are unaffected (Robichaud et al., 1980). The VP1 protein appears, like ABI3, to participate in mediating abscisic acid actions during seed development. Because vp1 mutants are also deficient in the synthesis of anthocyanins in the aleurone layer, a phenotype not observed in abscisic acid–deficient mutants (Robertson, 1955), the VP1 protein appears to fulfill additional roles less directly related to abscisic acid. Available evidence supports the possibility that the VP1 protein is a transcriptional activator that regulates the expression of both Em (an abscisic acid–regulated gene abundantly expressed during late embryogenesis) and C7 (a regulatory gene of the seed anthocyanin pathway). Indeed, overexpression of VP1 in maize protoplasts promotes transcriptional activation of coelectroporated Em-GUS (McCarty et al., 1991) or C7-GUS (Hattori et al., 1992) reporter genes. However, the cis-elements mediating VP1 and abscisic acid regulations are partially separable in the C7 promoter (Hattori et al., 1992).

McCarty et al. (1991) demonstrated that the NH2-terminal acidic portion of the VP1 protein is essential for transcriptional activation of the Em-GUS reporter gene by overexpressed VP1, and that it can be functionally replaced in this assay by the acidic domain of the herpes simplex virus VP16 transcription factor. The corresponding ABI3 region is poorly conserved at the amino acid level; however, it is globally acidic as well and may thus have similar functional properties. Alternatively, as mentioned above, the ABI3 protein contains several other regions that can potentially have transcriptional activation properties.

High sequence similarity between the ABI3 and VP1 proteins is found in three domains of these polypeptide chains. The region from ABI3 residues 255 to 310 was outlined by McCarty et al. (1991) as containing several positively charged stretches with helix-forming potential that might interact with DNA. The basic segment extending from ABI3 residues 439 to 476 contains the putative nuclear targeting signal described in this study, but might alternatively fulfill other functions. Most remarkable is the quasi-identity found from ABI3 residues 557 to 674, a region that is encoded by four conserved exons. This domain has characteristics similar to those of the DNA binding and dimerization domain of the human CTIF/NF1 transcriptional activator (Mermod et al., 1989); it has a net positive charge and its NH2-terminal portion can potentially form an amphipatic basic helix. Although binding of the VP1 protein to putative target DNA sequences has not been demonstrated so far (McCarty et al., 1991), the features of the conserved regions suggest that the ABI3 and VP1 proteins might actually contact DNA.

The described phenotypes of the abi3 and vp1 mutants suggest potentially interesting differences in the respective roles of the ABI3 and VP1 proteins. For instance, ABI3, but apparently not VP1, controls the sensitivity of seedling growth to abscisic acid (Koornneef et al., 1989; Finkelstein and Somerville, 1990).
of proteins. Availability of the cloned AB13 gene now opens
the functional counterpart of the maize
VP7, but not AB13, has been shown to
regulate anthocyanin production in the seed (McCarty et al.,
1990; Hattori et al., 1992). At this stage, it remains an open
question whether the AB13 protein in Arabidopsis is the exact
functional properties and physiological roles of the
AB13 protein.

METHODS

Plant Materials

Arabidopsis thaliana seed stocks were used as indicated. The
wild-type Columbia and C24 lines were provided by Frederick Ausubel (Mas-
sachusetts General Hospital, Boston); the Landsberg erecta wild-type,
hy2,gl7, and ab13 lines were provided by Maarten Koornneef (Agricul-
tural University, Wageningen, The Netherlands). The ab13-1 mutant
(isolation number CIV; Koornneef et al., 1984) was obtained by ethyl
methanesulfonate (EMS) treatment of Landsberg erecta seeds. The
ab13-4 mutant (isolation number sm1) was obtained by EMS treatment of
ab13-1 seeds; its phenotype closely resembles that of the ab13-3
(Nambara et al., 1992) allele (K. Léon and M. Koornneef, personal
communication).

Plants were routinely grown in a greenhouse (22°C, 16-hr pho-
toperiod), on soil irrigated with mineral nutrients. The
abi3-4 mutant allele was crossed to the wild-type Columbia ecotype, and F, progeny was allowed to self. Recombinants between the AB13 locus and the flanking marker were identified
in the F2 population by selecting plants that displayed the abb, but
and the hy2 (or

Isolation of Recombinant Plants

The abi3-1 mutant allele was crossed to a double mutant line contain-
ing both recessive morphological markers hy2 and gl7. The F1 plants
were allowed to self-pollinate, and the double mutants abi3abi3,hy2hy2
and abi3abi3gl7gl7 were isolated by screening the F2 populations.
Individuals homozygous for the abi3 mutation were selected for their
ability to germinate in the presence of abscisic acid (mixed isomers; Sigma); seeds were plated ei-
other on perlite with mineral nutrients (Koornneef et al., 1984) or surface
sterilized and plated on germination medium (Valvekens et al., 1988)
agar plates. Petri dishes were incubated 3 days in the dark at 4°C and
then in a growth chamber (21°C, 16-hr photoperiod).

cDNA Library Construction and Screening

Total RNA was isolated from green siliques (all developmental stages
were pooled) of the Columbia line, and poly(A)+ RNA was then puri-
fied by oligo(dT) cellulose chromatography (Ausubel et al., 1989).
Double-stranded cDNA was synthesized, using oligo(dT) as primer,
and washed in 0.1 x SSC, 0.1% SDS, and once with 0.1 x SSC, 0.1% SDS,
in a 5 to 20% sucrose gradient. Two pools were made that contained
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RFLP Analysis

All of the RFLP probes used here are cosmid clones derived from a
genomic library made from Columbia nuclear DNA (Nam et al., 1989)
with the exception of λbat 433, which was provided by Chang et al.
(1988). The restriction enzymes used for RFLP detection were: HindIII
(4711, 3669), XbaI (17287, λbat 433), Clal (6220), and BclI (6974). Total
genomic DNA extraction and DNA gel blot analysis were performed as
described by Nam et al. (1989).

Transgenic Plants

Restriction fragments of cosm id 4711 were gel purified and cloned into
the HindIII site of vector pGSFRI61 (Valvekens et al., 1988) by blunt-
end ligation. Recombinants were cloned into the Escherichia coli DH5α
strain, and then mobilized into the Agrobacterium tumefaciens
C58C1 rif (pGV2260) strain by triparental mating using E. coli K514 as
a helper strain. Agrobacterium transconjugants were selected on AT
sucrose plates containing rifampicin (75 µg/mL) and spectinomycin
(500 µg/mL). Structure of the cointegrates was controlled by DNA gel
blot analysis of total Agrobacterium DNA.

Arabidopsis root explants were transformed with Agrobacterium and
regenerated as described by Valvekens et al. (1988). Primary trans-
forms (T1) were grown in vitro in Magenta containers. For each line, T2
seeds were harvested aseptically and plated on 50 mg/L kanamy-
cin to assay the segregation ratio of kanamycin resistance, and the
ploidy level of T2 plants was measured by flow cytometry (Brown et
al., 1991). For selected transgenic lines, kanamycin-resistant T2 plants
were transferred to the greenhouse and crossed to the ab13-1 mutant.
DNA Sequence Analysis

Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (Ausubel et al., 1989) using Sequenase (United States Biochemical). Sequence of the cDNA clone was established by analyzing a series of ExolII deletion subclones and by using directed oligonucleotide primers. Sequence of the 4711-E genomic fragment was obtained by analyzing various restriction fragment subclones and by using directed oligonucleotide primers. Total genomic DNA was amplified by polymerase chain reaction (PCR) using primers carrying a Sal1 restriction site, and the PCR products were cloned into pBluescript SK− for sequencing. Putative mutations were then verified by directly sequencing the total PCR products derived from independent amplification reactions.

Sequence analyses were done using the Genetic Computer Group Sequence Analysis Software Package (Devereux et al., 1984). Sequence comparisons with data bases were performed using the FASTA program (Pearson and Lipman, 1988).

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