The Arabidopsis *ABSCISIC ACID–INSENSITIVE2 (ABI2)* and *ABI1* Genes Encode Homologous Protein Phosphatases 2C Involved in Abscisic Acid Signal Transduction

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Abscisic acid (ABA) mediates seed maturation and adaptive responses to environmental stress. In Arabidopsis, the *ABA-INSENSITIVE1* (*ABI1*) protein phosphatase 2C is required for proper ABA responsiveness both in seeds and in vegetative tissues. To determine whether the lack of recessive alleles at the corresponding locus could be explained by the existence of redundant genes, we initiated a search for *ABI1* homologs. One such homolog turned out to be the *ABI2* locus, whose *abi2-1* mutation was previously known to decrease ABA sensitivity. Whereas *abi7-7* is (semi)dominant, *abi2-7* has been described as recessive and maternally controlled at the germination stage. Unexpectedly, the sequence of the *abi2-7* mutation showed that it converts Gly-168 to Asp, which is precisely the same amino acid substitution found in *abi7-7* and at the coincidental position within the *ABI1* phosphatase domain (Gly-180 to Asp). In vitro assays and functional complementation studies in yeast confirmed that the *ABI2* protein is an active protein phosphatase 2C and that the *abi2-7* mutation reduced phosphatase activity as well as affinity to Mg²⁺. Although a number of differences between the two mutants in adaptive responses to stress have been reported, quantitative comparisons of other major phenotypes showed that the effects of both *abi1-1* and *abi2-1* on these processes are nearly indistinguishable. Thus, the homologous *ABI1* and *ABI2* phosphatases appear to assume partially redundant functions in ABA signaling, which may provide a mechanism to maintain informational homeostasis.

**INTRODUCTION**

Abscisic acid (ABA) regulates many aspects of plant growth and development, including seed maturation and dormancy as well as tolerance to adverse environmental conditions (Chandler and Robertson, 1994; Giraudat et al., 1994; Ward et al., 1995). On the basis of physiological and molecular criteria, the action of ABA can range from the rapid modification of ion fluxes in stomatal guard cells (Ward et al., 1995) to more long-term effects involving changes in gene expression patterns (Chandler and Robertson, 1994). Studies of guard cells have provided evidence for multiple ABA perception sites that are distributed both inside (Allan et al., 1994; Schwartz et al., 1994) and outside (Anderson et al., 1994) of the cell. Whether this represents a canonical model of ABA perception applicable to other cell types is currently not known. Furthermore, the molecular identity of these perception sites and the way the ABA signal is subsequently transduced remain unclear.

The isolation of Arabidopsis mutants with altered ABA responsiveness has led to the identification of several likely key intermediates in these signal transduction chains. Mutations at the *ABA-INSENSITIVE3 (ABI3)* (Koornneef et al., 1984), *ABI4*, and *ABI5* (Finkelstein, 1994a) loci and the enhanced response to the ABA *ERA7* (Cutler et al., 1996) locus affect seed-specific developmental processes. Two of these genes have been cloned (Giraudat et al., 1992; Cutler et al., 1996). The *ABI3* gene (Giraudat et al., 1992), by homology to the maize *VIVIPAROUS1* gene (McCarty et al., 1991), encodes a putative transcription factor essential for mediating a subset of the seed developmental programs (Ooms et al., 1993; Parcy et al., 1994; Nambara et al., 1995). This is corroborated by ectopic expression of the wild-type *ABI3*, which rendered vegetative tissues hypersensitive to ABA and activated expression of several otherwise seed-specific genes in leaves when exogenous ABA was supplied (Parcy et al., 1994). The *ERA1* gene, which encodes the β subunit of a farnesyl transferase, has been postulated to be a negative regulator of embryonic ABA signals involved in seed dormancy, probably by modifying several signal transduction proteins for membrane localization (Cutler et al., 1996).

In contrast, the *abi1* and *abi2* mutations are the most pleiotropic. They affect both seeds and vegetative tissues, suggesting that their gene products act in ABA signal transduction...
before major branch points that control tissue-specific cascades. Differential effects imparted by the abi1-1 and abi2-1 mutations are most evident in adaptive responses to drought by modifications of the root, a process known as drought rhizogenesis (Vartanian et al., 1994), and induction of certain genes (Gilmour and Thomashow, 1991; Gosti et al., 1995; de Bruxelles et al., 1996; Soderman et al., 1996). Nevertheless, these mutants also have several phenotypes in common. In seed, both mutations reduce dormancy and the sensitivity of germination to the inhibitory effects of ABA (Koornneef et al., 1984; Finkelstein and Somerville, 1990). In vegetative tissues, both mutants display ABA-resistant seedling growth, abnormal stomatal regulation, and defects in various ABA-induced morphological and molecular responses (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Schnall and Quatrano, 1992; Finkelstein, 1994b; Gosti et al., 1995).

The cloning and characterization of the ABI1 gene (Leung et al., 1994; Meyer et al., 1994) revealed that it encodes a protein composed of a novel N-terminal segment and a C-terminal domain that is an active protein serine/threonine phosphatase 2C (PP2C) (Bertauche et al., 1996). In guard cells, ABI1 appears to relay, together with counteracting protein kinases, ABA signals to stomatal regulation in that defects in stomatal closure caused by the abi1-1 mutant gene can be partially suppressed by kinase antagonists (Armstrong et al., 1995). The abi1-1 mutation, converting Gly-180 to Asp in the PP2C domain, leads to a marked decrease in the phosphatase activity of ABI1 (Bertauche et al., 1996). However, this mutation behaves genetically, depending on the particular phenotypes, as either fully dominant or semidominant (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Schnall and Quatrano, 1992; Finkelstein, 1994b; Gosti et al., 1995).

Equally striking is that no recessive mutant alleles have yet been described for the A617 locus. This latter observation raises the possibility that ABA signaling may involve additional compensatory pathways, possibly represented by ABI1 homologs.

We report here the isolation and characterization of one such ABI1 homolog. This homolog turned out to be the ABI2 gene mentioned above. The molecular data combined with our quantitative phenotypic comparisons of the abi1 and abi2 mutants strongly suggest that the roles of ABI1 and ABI2 in mediating ABA signals are mechanistically much more similar than previously anticipated. The concerted action of these two PP2Cs could reflect a versatile control mechanism in response to complex cellular and environmental cues.

RESULTS

Isolation of an ABI1 Homolog

To search for ABI1 homologs, the entire coding region of the ABI1 cDNA was used initially to screen under reduced stringency (see Methods) an Arabidopsis genomic library constructed in yeast artificial chromosomes (YACs) (Creusot et al., 1995). In addition to those YACs containing the ABI1 gene (CIC1E5, CIC1G9, CIC3C11, CIC10G2, CIC12B3, and CIC12H1), we detected three others that subsequently were found to hybridize only with cDNA probes containing the phosphatase but not the N-terminal domain of ABI1. These three YACs (CIC8C9, CIC9E2, and CIC10B4) belong to an existing contig on the bottom of chromosome 5 as determined by their cohybridization with the restriction fragment length polymorphism markers m558A and m70 (D. Bouchez, personal communication; see Methods).

The YAC CIC10B4 was converted into cosmid clones and screened with the entire ABI1 cDNA coding region under the same reduced stringency. The region containing ABI1 homology in one of the cosmids (cos27-1) was isolated as a 4.2-kb HindIII-XbaI restriction fragment and was in turn used to identify specific cDNAs. Two positive clones were obtained from among 5 x 10⁵ candidates screened, and the longer cDNA with a 1.6-kb insert was retained for subsequent studies. This cDNA was named pcABI2 because further analysis revealed that this ABI1 homolog corresponded to the ABI2 locus (see below).

As shown in Figure 1A, DNA sequence determination of the insert in pcABI2 predicts a 423-amino acid polypeptide. Figure 2 shows that the ABI2 protein has the same modular architecture as ABI1. The C-terminal region of the ABI2 protein (residues 90 to 423) is homologous to PP2Cs (34% identity with the yeast PTC1 protein) and is most closely related (86% identity) to the C-terminal PP2C domain of ABI1 (residues 106 to 434). The ABI2 protein also has an N-terminal segment (residues 1 to 89) that displays no extensive similarity with available protein sequences other than the N-terminal domain of ABI1 (residues 1 to 105). The N-terminal domain of ABI2, however, is less conserved (49% identity) and shorter by 16 amino acids compared with its counterpart in ABI1.

The ABI1 Homolog Is ABI2

The structural similarity between the above-mentioned homolog and the ABI1 protein prompted us to examine whether this homolog could in fact be ABI2. The abi1 and abi2 mutants share several common phenotypes (Koornneef et al., 1984; Finkelstein and Somerville, 1990); moreover, abi2 is the only described ABA-insensitive mutation mapping, like the ABI1 homolog (see above), to the bottom of chromosome 5 (see Methods).

The abi1-1 mutation is a G-to-A transition (at nucleotide 970 in the cDNA pcABI1-c38; EMBL accession number X77116) that destroys a diagnostic Ncol restriction site present in the wild-type ABI1 gene and that is located in a region encoding amino acids highly conserved among PP2Cs (Figure 2; Leung et al., 1994; Meyer et al., 1994). In view of the common phenotypes between the two mutants, we
tested whether the abi2-1 mutation could be located in the same stretch of nucleotides (which also contains a Ncol site CCATGG beginning at nucleotide 548 in Figure 1) and thereby disrupt the normal functions mediated by this conserved protein subdomain in a similar manner.

Figments of the ABI2 gene encompassing this restriction site were amplified by polymerase chain reaction (PCR) from genomic DNA of both the wild type and the mutant abi2-1 (both in the Landsberg erecta ecotype). As shown in Figure 3, ABI2 fragments derived from the abi2-1 mutant were indeed consistently found to be resistant to digestion with Ncol. The entire genomic nucleotide sequences of both the wild-type ABI2 and mutant abi2-1 genes were then determined. The organization of the ABI2 gene is schematically depicted in Figure 1B. The only difference between the wild-type and mutant genes is a single base pair change in the above-mentioned Ncol site. The abi2-1 mutation is a G-to-A transition at position 553 in the cDNA sequence and converts Gly-168 to Asp. The entire genomic nucleotide sequences of both the wild-type ABI2 and mutant abi2-1 genes were then determined. The organization of the ABI2 gene is schematically depicted in Figure 1B. The only difference between the wild-type and mutant genes is a single base pair change in the above-mentioned Ncol site. The abi2-1 mutation is a G-to-A transition at position 553 in the cDNA sequence and converts Gly-168 to Asp.

To extend these molecular data, we used functional assays in transgenic plants to ascertain that this mutation was in fact responsible for the characteristic phenotypes of the abi2-1 mutant. In a first set of experiments, a cosmid clone (cosabi2) containing the mutant abi2-1 gene was isolated and introduced into Arabidopsis wild type (ecotype C24) by infiltration of plants with Agrobacterium (Bechtold et al., 1993). Three independent primary transformants (T0 plants) were obtained from scoring ~100,000 seeds derived from these infiltrated plants. Table 1 shows that like the original abi2-1 mutant (Koornneef et al., 1984), these three transformants displayed reduced seed dormancy in that in the absence of prior stratification, the germination rate of freshly harvested T1 seed progeny (which segregated for the transgene) was markedly higher than that of the wild type. Like the abi2-1 mutant, T1 seeds derived from line A also exhibited significant resistance to 3 µM exogenous ABA (Table 1), and all of the ABA-resistant seedlings tested (14 of 14) were resistant to kanamycin (selection marker for the T-DNA transgene) (data not shown). However, ABA resistance was
not detected in lines B and C, and none of the three transformants showed enhanced water loss from detached rosette leaves (Table 1), suggesting that the abi2-1 transgene was only weakly expressed.

We thus attempted in a second series of experiments to favor the recovery of transgenic plants with enhanced expression of the mutant ato/2-7 gene. The Gly-168-to-Asp mutation was re-created in the wild-type cDNA pcABI2 by site-directed mutagenesis. The insert from the mutated derivative pcabi2-1 was placed under the control of the double-enhanced cauliflower mosaic virus 35S promoter, and this construct (EN35S::abi2) was introduced into wild-type Arabidopsis plants. Eleven independent primary transformants (T0 plants) were obtained, but nine of them died from complete desiccation within a few days after their transfer from agar plates to soil. Like the original afo/2-7 mutant (Koornneef et al., 1984), the remaining viable transformants (lines 3 and 6) displayed an increased tendency to wilt and, as shown in Table 1, an enhanced water loss from detached rosette leaves as well as reduced seed dormancy. Line 3 also showed decreased sensitivity to the ABA inhibition of seed germination (Table 1), and all the ABA-resistant T1 seedlings tested (29 of 29) were resistant to kanamycin (data not shown).

The combined results with cosabi2 and EN35S::abi2 transgenic plants support the idea that transgenes carrying the Gly-168-to-Asp mutation were indeed capable of induc-
Table 1. Characteristics of Wild-Type Plants Transformed with the Mutant abi2-1 Gene or cDNA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Kanamycin Resistancea (%)</th>
<th>Seed Dormancyb (%)</th>
<th>Transpirationc</th>
<th>Germination on ABAd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>5</td>
<td>17.23 ± 0.81</td>
<td>0</td>
</tr>
<tr>
<td>abi2-1 mutant</td>
<td>0</td>
<td>100</td>
<td>51.15 ± 5.80a</td>
<td>100</td>
</tr>
<tr>
<td>cosabi2 line A</td>
<td>70</td>
<td>39</td>
<td>14.77 ± 0.76</td>
<td>46</td>
</tr>
<tr>
<td>cosabi2 line B</td>
<td>95</td>
<td>70</td>
<td>16.09 ± 0.82</td>
<td>0</td>
</tr>
<tr>
<td>cosabi2 line C</td>
<td>98</td>
<td>42</td>
<td>13.88 ± 0.89</td>
<td>0</td>
</tr>
<tr>
<td>EN35S::abi2 line 3</td>
<td>87</td>
<td>80</td>
<td>29.15 ± 0.67a</td>
<td>81</td>
</tr>
<tr>
<td>EN35S::abi2 line 6</td>
<td>94</td>
<td>49</td>
<td>24.53 ± 0.85a</td>
<td>0</td>
</tr>
</tbody>
</table>

a Percentage of T\(_t\) seeds that germinated on 50 mg/L kanamycin.
b Percentage of freshly harvested T\(_t\) seeds that germinated after 5 days. In similar experiments in which seeds were first incubated in the cold to break dormancy, 100% germination was observed for all genotypes (data not shown).
c Water loss from detached leaves of T\(_o\) plants. Values shown are the percentages of initial fresh weight lost after 2 hr. Mean ± se of measurements with two separate leaves.
d Percentage of T\(_t\) seeds that germinated after 3 days on 3 \(\mu\)M ABA.

*P \(=\) 0.025, two-sample unpaired Student's t test.

PP2C Activity of ABI2

To examine whether ABI2 has in vivo protein phosphatase activity, we performed complementation studies of a yeast mutant (Figure 4A). In yeast, the PTC1 gene encodes a functional PP2C, the disruption of which leads to a temperature-sensitive growth defect in that mutant cells grow slower at 37°C than at 30°C (Maeda et al., 1993; Figure 4A). The wild-type and mutant ABI2 cDNA were placed under the control of the alcohol dehydrogenase ADH1 yeast promoter in the expression vector p181ANE, and the constructs were introduced into the ptc1Δ yeast mutant TM126. The construct containing the wild-type ABI2 but not the mutant abi2 gene was able to reverse the temperature-sensitive growth defect of ptc1Δ (Figure 4A), which indicates that ABI2 can functionally compensate for the disrupted yeast PP2C.

The wild-type ABI2 and mutant abi2 were also expressed in Escherichia coli as fusion proteins with glutathione S-transferase (GST). The resulting products, GST-ABI2 and GST-abi2, were tested for their ability to dephosphorylate 32P-labeled casein, a commonly used artificial substrate in assaying PP2C activity (MacKintosh, 1993). Both GST-ABI2 and GST-abi2 were indeed capable of dephosphorylating casein (Figure 4B). However, the Gly-168→Asp substitution of the abi2-1 mutation in the PP2C domain significantly decreased this phosphatase activity (Figure 4B), which is consistent with the failure of abi2 to compensate for the ptc1Δ mutation in yeast (Figure 4A). The phosphatase activity of both recombinant proteins was diagnostic of PP2C (Cohen, 1989; MacKintosh, 1993) in that it was dependent on the presence of Mg\(^{2+}\) (Figure 4C) and unaffected by 10 \(\mu\)M okadaic acid (data not shown). However, the amino acid substitution in abi2-1 appears to have a negative influence on the protein's divalent cation affinity because GST-abi2 required higher Mg\(^{2+}\) concentrations with respect to its optimal activity relative to that of GST-ABI2 (Figure 4C).

Comparative Analysis of abi1-1 and abi2-1 Mutants

The discovery that ABI1 and ABI2 are homologous genes and that equivalent amino acid substitutions are found in the abi1-1 and abi2-1 mutant proteins prompted us to assess more critically the phenotypic similarity between these mutants. We thus quantified, side by side, the relative impact of the abi1-1 and abi2-1 mutations on several characteristic ABA responses.

As shown in Figure 5, analysis of homozygous mutants revealed that these two mutations had identical effects on ABA sensitivity of seed germination (Figure 5A), on stomatal regulation (Figure 5C), and on the induction of the RAB18 transcript by ABA (Figure 5D). Both mutations also reduced ABA sensitivity of root growth; however, abi1-1 was slightly more resistant than was abi2-1 (Figure 5B).

As shown in Figure 6A, the ABI2 transcript was detectable, like ABI1, in all of the plant organs examined. However,
Figure 4. PP2C Activity of ABI2 and Impact of the ab/2-1 Mutation.

(A) Complementation studies of the ptc1A yeast mutant TM126. For each strain, ~10^4 cells were spotted as 1-μL droplets onto agar plates containing synthetic complete medium lacking leucine and uracil and were incubated at either 30 or 37°C for 2 days. In each row, the yeast strain is the mutant TM126 transformed with the vector p181ANE as a control, pDBC1 containing the wild-type yeast PTC1 gene (Maeda et al., 1993), and p181ANE containing either the Arabidopsis wild-type (pADH-ABI2) or mutant (pADH-abi2) ABI2 gene. Similar results were obtained for five independent transformants for each of these constructs; only one representative sample for each construct is shown.

(B) In vitro protein phosphatase activity of GST-ABI2 and GST-abi2. Recombinant proteins (50 ng) were incubated at 30°C with 32P-labeled casein for the indicated times in the presence of 20 mM magnesium acetate. In experiments with 20 ng of recombinant proteins, the initial rate of 32Pi release was ~10-fold lower with GST-abi2 than with GST-ABI2 (data not shown).

(C) Magnesium dependency of protein phosphatase activities. Reactions were performed with 25 ng of GST-ABI2 for 20 min (left) or 90 ng of GST-abi2 for 40 min (right) at 30°C with the indicated concentrations of magnesium acetate (Mg^{2+}). The percentage of 32Pi released is shown in different scales for GST-ABI2 and GST-abi2. Phosphatase activities of both proteins are absolutely dependent on the presence of Mg^{2+}. However, the activity of GST-ABI2 was maximal at 2 mM Mg^{2+}, whereas the activity of GST-abi2 progressively increased commensurate with the Mg^{2+} concentrations and was maximal at 10 mM Mg^{2+}.

DISCUSSION

Among the various ABA sensitivity mutations characterized so far in Arabidopsis (Koornneef et al., 1984; Finkelstein, 1994a; Cutler et al., 1996), only ab/7-7 and ab/2-7 affect ABA responsiveness in both seeds and vegetative tissues. Our study demonstrates that the corresponding ABI1 and ABI2 genes are in fact homologous to each other. In particular, the predicted ABI1 and ABI2 proteins share the same modular architecture composed of a C-terminal domain with PP2C activity and a characteristic N-terminal domain that displays no extensive similarity to other known proteins.

The ethyl methanesulfonate-induced ab/7-7 and ab/2-7 mutants, which both have been isolated on the basis of decreased ABA sensitivity (Koornneef et al., 1984), carry identical Gly-to-Asp substitutions at equivalent positions in the ABI1 (Gly-180) and ABI2 (Gly-168) phosphatase domains. These Gly-to-Asp substitutions occur at positions coincident to Ala-63 of the human PP2Cα, the crystal structure of which has been determined recently (Das et al., 1996). Such a substitution has been predicted to disrupt the con-
Figure 5. Phenotypic Comparison of the abi1-1 and abi2-1 Mutants.

ABA responses were analyzed for plant genotypes that were all in the Landsberg erecta background: wild type (Ler), homozygous mutants abi1-1 (abi1) and abi2-1 (abi2), and heterozygous F1 progeny from Ler × abi1 and Ler × abi2 crosses.

(A) ABA dose response for germination inhibition. Seeds were plated on medium supplemented with the indicated concentration of ABA (mixed isomers), chilled for 3 days at 4°C in darkness, and incubated for 3 days at 21°C with a 16-hr-light photoperiod. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (40 to 80).

(B) Inhibition of root growth. Seeds were germinated and grown for 5 days on ABA-free medium. These seedlings were then incubated vertically on medium supplemented with the indicated concentrations of ABA, and their root length was scored after 4 days. Root growth of ABA-treated seedlings is expressed as a percentage relative to controls incubated on ABA-free medium. Values shown are mean ± SE from samples composed of 11 to 15 seedlings each.

(C) Kinetics of water loss from excised leaves. Three to four young rosette leaves (0.5 to 1 cm²) were excised from the same plant, and their total fresh weight was measured at different time points during incubation at ambient laboratory conditions. Water loss is expressed as the percentage of initial fresh weight. Values shown are mean ± SD of measurements with five individual plants per genotype.

(D) ABA induction of the RAB18 transcript. Total RNA was extracted from plantlets grown for 5 days on ABA-free medium and then transferred to medium containing the indicated concentrations of ABA and incubated for 55 hr. RNA blots were hybridized with the RAB18 probe and then with an 18S rRNA probe for normalization. RAB18 mRNA levels are expressed in arbitrary units, with 1 unit corresponding to the mRNA level in wild-type (Ler) plantlets on ABA-free medium.

formation of the neighboring metal-coordinating residues Asp-60 and Gly-61 (equivalent to Asp-177/Gly-178 of ABI1 and Asp-165/Gly-166 of AB12), with a concomitant reduction in catalytic activity (Das et al., 1996). The abi1-1 and abi2-1 mutations did indeed similarly entail a marked decrease in the PP2C activity of the ABI1 (Bertauche et al., 1996) and ABI2 (Figure 4B) proteins, respectively. Furthermore, the mutant GST-abi2 fusion protein required higher Mg²⁺ concentrations with respect to its optimal activity compared with wild-type GST-ABI2 (Figure 4C), suggesting that the Gly-168-to-Asp substitution in abi2-1 effectively decreases divalent cation affinity.
Figure 6. RNA Gel Blot Analyses of ABI1 and ABI2 Expression.

(A) Comparison of the expression profiles of the ABI1 and ABI2 mRNAs. Total RNA was extracted from the indicated organs of wild-type plants grown in the greenhouse, except for roots, which were derived from 10-day-old plants grown in vitro (Parcy et al., 1994). Duplicate sets of the samples (10 μg of total RNA) were resolved by electrophoresis on the same gel and then transferred to the same membrane. After transfer, the membrane was bisected, and each half was hybridized to either an ABI1 (top) or an ABI2 (bottom) gene-specific probe. The RNA blot hybridized with the ABI2 probe required a longer exposure to achieve approximately the same signal intensity as that observed for the ABI1 RNA blot. The generally lower expression level of the ABI2 transcript is consistent with the lower frequency (approximately threefold) at which ABI2 clones, relative to those for ABI1, were identified in the same cDNA library. A higher molecular weight band was detected with both ABI1 probes in RNA isolated from green siliques, which might be an artifact due to polysaccharide contaminants in the samples.

(B) Sorbitol induction of the ABI1 and ABI2 mRNAs. Plantlets from the wild type (Ler) and from the ABA biosynthetic mutant aba1-1 (abal) (Koornneef et al., 1982) were grown for 10 days on germination medium (Parcy et al., 1994), transferred to the same medium containing (+) or not (−) a final concentration of 10% (w/v) sorbitol, and incubated for 3 days. The aerial parts from these plantlets were then harvested and subjected to RNA gel blot analysis.

(C) ABA induction of the ABI1 and ABI2 transcripts. Total RNA was extracted from plantlets grown for 5 days on ABA-free medium, transferred to medium containing the indicated concentrations of ABA, and incubated for 55 hr. RNA blots were hybridized with ABI1 (left) and ABI2 (right) gene-specific probes and then with an 18S rRNA probe for normalization. ABI1 mRNA levels are expressed in arbitrary units, with 1 unit corresponding to the mRNA level in wild-type (Ler) plantlets on ABA-free medium.

abi1-1 and abi2-1 Are (Semi)Dominant and Zygotic Mutations

The effects of the abi1-1 and abi2-1 mutations on ABA sensitivity of seed germination have been reported to range from recessive to nearly fully dominant, depending on whether these mutant alleles were inherited paternally or maternally (Finkelstein, 1994b). In contrast, in our germination tests, these mutations were fully dominant over their wild-type allele irrespective of the parental origin (Figure 5A). That is, heterozygotes that inherited the mutant allele paternally or maternally displayed the same degree of ABA resistance as homozygous mutants, suggesting that sensitivity of seeds to applied ABA is determined by the zygotic genotype rather than maternally controlled. We do not know the exact reason for the differences between the two sets of experiments. However, we have not investigated whether variations in the environmental conditions used for growing the parental plants could change the degree of ABA resistance of the derived heterozygous seed progeny.

On the basis of ABA sensitivity of seedling growth, abi1-1 originally was described as a dominant mutation, whereas abi2-1 was described as nearly completely recessive (Koornneef et al., 1984). As discussed above, both mutations were found here to be fully dominant in reducing ABA sensitivity of seed germination. In addition, both mutations are semidominant in reducing ABA sensitivity of root growth (Figure 5B) and in altering stomatal regulation (Finkelstein, 1994b). Thus, the degree of dominance for both abi1-1 and abi2-1 is contingent on the particular phenotypes. In light of the current molecular data, the similar degree of (semi)dominance observed here for abi1-1 and abi2-1 is consistent with the finding that these mutations entail identical amino acid changes in homologous genes. The ability of the abi2-1 cDNA to provoke abnormalities in dormancy and transpiration in transgenic wild-type plants also reinforces the interpretation that this mutation is not recessive (Table 1).

Possible Molecular Mechanisms

The abi1-1 and abi2-1 mutants displayed several common phenotypes (decreased sensitivity to the ABA inhibition of seed germination and seedling growth, altered stomatal regulation, and decreased responsiveness of the RAB18 transcript to applied ABA), which are comparable not only qualitatively but quantitatively (Figure 5). These quantitative
similarities argue that, as schematically depicted in Figure 7, the homologous ABI1 and ABI2 proteins have largely overlapping functions in ABA signaling.

For example, the ABI1 and ABI2 phosphatases might recognize common physiological substrates involved in the ABA responses delineated by the above-mentioned mutant phenotypes. This hypothesis would be consistent with the observation that in germination assays, the abi1-1 abi2-1 double mutant is not more resistant to ABA than are the parental single mutants (Finkelstein and Somerville, 1990). Furthermore, their overlapping functions provide a simple explanation why recessive alleles have not been identified at the ABI1 and ABI2 loci on the basis of seed germination on ABA. In this regard, ABA signaling is reminiscent of the ethylene response pathway in which ETR1 and its homolog ERS have been proposed to act as redundant hormone sensors and in which only semidominant alleles have been recovered at the ETR1 locus (Hua et al., 1995).

However, in the case of the two ABI loci, it is unclear why no dominant mutations other than the same G-to-A transi-

**Figure 7. A Model for the Respective Roles of ABI1 and ABI2 in ABA Signaling.**

Phenotypic analyses of the abi1-1 and abi2-1 mutants indicate that the ABI1 and ABI2 protein phosphatases have overlapping functions in mediating several responses to either endogenous ABA (promotion of seed dormancy and stomatal closure) or exogenous ABA (inhibition of seed germination and seedling growth; induction of the RAB18 transcript). However, induction of the Adh transcript by ABA and drought stress is severely inhibited in abi2-1 and not in abi1-1 (de Bruxelles et al., 1996). Conversely, drought rhizogenesis (Vartanian et al., 1996), induction of the ATHB-7 transcript (Gostì et al., 1994), induction of the ATHB-7 transcript by water stress (Söderman et al., 1996), as well as the induction of several cold-responsive COR genes by exogenous ABA (Gilmour and Thomashow, 1991) are affected in abi1-1 but not in abi2-1. Also, inductions of the ABI1 and ABI2 transcripts themselves by applied ABA are inhibited in abi1-1 and abi2-1, suggesting that in the simplest scheme, both proteins may impinge on the effector(s) that participates in an autoregulatory circuit required to maintain proper levels of the two ABI transcripts.
**METHODS**

**Gene Cloning**

The *Arabidopsis thaliana* CIC library constructed in yeast artificial chromosomes (YACs) (Creusot et al., 1995) was screened essentially as described by Leung et al. (1994). DNA probes consisting of the entire coding region of abscisic acid (ABA)-insensitive *ABI1* (nucleotides 239 to 1926), the phosphatase 2C (PP2C) domain (nucleotides 711 to 1926), or the N-terminal domain (nucleotides 239 to 710) were isolated from the cDNA pcABI2-c38 (Leung et al., 1994) as restriction fragments. After hybridization with radiolabeled probes overnight at 65°C (Church and Gilbert, 1984), final washes of the filters were done in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS either at 65°C, conditions that did not permit detection of cross-hybridization with homologous genes (stringent conditions), or at 55°C (reduced stringency). The YAC's contig, which includes CIC9C9, CIC9E2, and CIC1094 on chromosome 5, has been described by R. Schmidt (http://genome-www.stanford.edu/Arabidopsis/locus.html). The ab2-1 mutation has been mapped to the bottom of chromosome 5 by R. Finkelstein (Linkage Table for Mutant Genes, http://mutant.lse.okstate.edu/classical_map.html).

Total genomic DNA was isolated from yeast containing YAC CIC10B4. After digesting the DNA partially with MboI, the ends of the fragments were incompletely filled in with dgTTP and daTTP. DNA fragments were then fractionated by centrifugation in a linear 1.25 to 5.0 M NaCl gradient, and DNA in the 20-kb range was ligated into the plasmid generated by exonuclease 111 and Sh11 ligation (Sequencher, version 2.2; Gene Vie Inc., Foster City, CA). Synthetic oligonucleotides used for screening of the genomic library and for PCR amplification of the *ABI1* gene sequence were 5'-CATCATCTGCTATGGCAGG-3' and 5'-CCGGAGCATGAGCCACAG-3'. PCR reactions were performed in 50 µL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 0.2 mM each dNTP, 100 ng of template DNA, 50 ng of each primer, and 2.5 U of Taq polymerase (Appligene Oncor, Illkirch, France). Amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

**DNA Sequencing**

Double-stranded DNA was sequenced on an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). The sequence of pcABI2 cDNA was determined from deletion derivatives of the plasmid generated by exonuclease III and S1 nuclease digestion. Overlapping genomic fragments encompassing the entire *ABI2* gene were obtained both from wild-type and ab2-1 DNA by polymerase chain reaction (PCR), using specific primers. The amplified products were sequenced directly on both strands, using appropriate primers. Sequence analyses and alignments were done with programs of the Genetics Computer Group (Madison, WI) software package (Devereux et al., 1984).

**Site-Directed Mutagenesis of the ABI2 cDNA**

A cDNA for the *abi2-1* mutant gene was created by site-directed mutagenesis of the wild-type pcABI2 cDNA, as described previously (Bertauche et al., 1996), by using the mutagenic oligonucleotide primer 5'-GCATGACAGTCTCAG-3', creating a G-to-A change at nucleotide 553 in pcABI2. A positive clone (pcabi2-1) was verified by sequencing and retained for subsequent use.

**Production and Characterization of Transgenic Plants**

The mutant cDNA insert was excised from pcabi2-1 with Smal and cloned into the Smal site of the binary plant transformation vector pDE1070 (Parcy et al., 1994). The construct was introduced into the *Agrobacterium tumefaciens* C58C1(RifGv2260) by electroporation (Wen-Jun and Forde, 1989). The cos左侧 cosmid containing the mutant *abi2-1* gene was introduced into the E. coli ST17-1 (Simon et al., 1983) by electroporation and then mobilized into the *Agrobacterium* pGV3101 (pMP90RK) by bacterial conjugation (Koncz and Schell, 1988).

Wild-type plants (ecotype C24) were inoculated by vacuum infiltration (Bechtold et al., 1993) with the above-mentioned agrobacteria, and transformed seeds were selected on kanamycin (50 µg/mL) (Parcy et al., 1994). For comparing stomatal regulation in transgenic and control wild-type plants, two detached rosette leaves per individual plant were incubated abaxial face up at ambient laboratory conditions, and their water loss was measured after 2 h. To test dormancy, mature seeds from transgenic plants and control untransformed plants were harvested at the same time. Approximately 50 seeds were immediately surface sterilized, plated on germination medium, and incubated at 21°C with a 16-hr-light photoperiod (Parcy et al., 1994). As controls for seed viability, samples (50 seeds) from the same batches of seeds were first stratified (i.e., plates were chilled for 4 days at 4°C in darkness) to break seed dormancy and then incubated at 21°C. To test ABA resistance, 50 to 90 seeds were plated on germination medium containing 3 mM ABA, stratified, and then incubated at 21°C for 3 days. Randomly selected germinated seeds (emerged radicle and green cotyledons) were then transferred to germination medium containing 50 µg/mL kanamycin to assay the presence of the transgene. At the same time, seeds of the same transgenic lines were plated directly onto germination medium containing 50 µg/mL kanamycin.

**Determining Genotypes of Plants at the ABI1 and ABI2 Loci**

Total genomic DNA was extracted from one or two rosette leaves (Konieczny and Ausubel, 1993). The oligonucleotide primers used for amplification of the *ABI1* gene fragment were 5'-CATATCCTCGCGCCGAGAT-3' and 5'-CCATTCCACGTACTTCTTCTAC-3'. The oligonucleotide primers used for amplification of the *ABI2* gene fragment were 5'-CATCATCTGCTTGGACAG-3' and 5'-CCGGAGCATGAGCCAACAG-3'. PCR reactions were performed in 30 µL containing 10 ng of genomic DNA, 500 ng of each primer, and 1 unit of Taq polymerase (Appligene Oncor, Illkirch, France). Amplification conditions were 94°C for 180 sec followed by 30 cycles consisting of 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec.
The coding regions of the wild-type ABI2 (pcABI2) and of the mutant abi2-1 (pcabi2-1) cDNAs were amplified by PCR by using the Pfu polymerase and primers 5'-GTTGAATTCATATGGACGAATGTTTCC-3' and 5'-GTTGAATTCATATGCCCTCACTAAAG-3'. The amplification products were then digested with EcoRI and cloned into the EcoRI site of the yeast expression vector p181ANE (generously provided by W. Frommer, Institut für Genbiologische Forschung, Berlin, Germany), which contains a 2-μm origin of replication, the LEU2 selectable marker, and the strong alcohol dehydrogenase promoter ADH1 for heterologous gene expression. Constructs were verified by sequencing and introduced into the yeast mutant TM126 in which PTC1 is mutated by insertion of a URA3 marker (~tclh) (Maeda et al., 1993). Selection of transformants and complementation assays were performed as previously described (Bertauche et al., 1996).

The EcoRI fragments containing the wild-type ABI2 and mutant abi2-1 coding regions (see above) were cloned into the EcoRI site of the XgtlO cDNA library; Plant Genetic Systems for the T-DNA vector pDBCl and the yeast strain TM126; Wolfgang Frommer for the vector p181ANE; Clare Lister and Caroline Dean for the vector and map of pDClO4541; Andreas Bachmair for the xgtl0 cdNA library; Plant Genetic Systems for the T-DNA vector pDE1000 used to construct pDE1070; Liliane Troussard, Rémy Drouen, and Nathalie Mansion for technical assistance; and Jean-Claude Barbet for assistance with computer analyses. This work was supported in part by the Centre National de la Recherche Scientifique, the Ministère de l’Enseignement Supérieur et de la Recherche, the International Human Frontier Science Program (RG-303/95), and the European Community BIOTECH program (Grant No. Bi04-CT96-0062).

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The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI11 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction.

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