The Plant Cell, Vol. 9, 759–771, May 1997 © 1997 American Society of Plant Physiologists

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

Jeffrey Leung,[1] Sylvain Merlot,[1] and Jérôme Giraudat[2]

Institut des Sciences Végétales, Centre National de la Recherche Scientifique UPR40, avenue de la Terrasse, 91190 Gif-sur-Yvette, France

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 *abi1-1* is (semi)dominant, *abi2-1* has been described as recessive and maternally controlled at the germination stage. Unexpectedly, the sequence of the *abi2-1* mutation showed that it converts Gly-168 to Asp, which is precisely the same amino acid substitution found in *abi1-1* 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 *abi2-1* mutation reduced phosphatase activity as well as affinity to Mg^{2+}. 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 *ERA1* (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

[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed. E-mail jerome.giraudat@isv.cnrs-gif.fr; fax 33-1-69-82-36-95.

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; Söderman 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, 1994b). Equally striking is that no recessive mutant alleles have yet been described for the *ABI1* 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 (CIC9C9, 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 mi70 (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 5×10^5 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 NcoI 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

A

```
ttttgttaaagttcaagaaagttctttttttctttttttttcctcctttaATGGACGAAG  60
                                                  M  D  E  V
TTTCTCCTGCAGTCGCTGTTCCATTCAGACCATTCACTGACCCTCACGCCGGACTTAGAG  120
 S  P  A  V  A  V  P  F  R  P  F  T  D  P  H  A  G  L  R  G
GCTATTGCAACGGTGAATCTAGGGTTACTTTACCGGAAAGTTCTTGTTCTGGCGACGGAG  180
 Y  C  N  G  E  S  R  V  T  L  P  E  S  S  C  S  G  D  G  A
CTATGAAAGATTCTTCCTTTGAGATCAATACAAGACAAGATTCATTGACATCATCATCAT  240
 M  K  D  S  S  F  E  I  N  T  R  Q  D  S  L  T  S  S  S  S
CTGCTATGGCAGGTGTGGATATCTCCGCCGGAGATGAAATCAACGGTTCAGATGAGTTTG  300
 A  M  A  G  V  D  I  S  A  G  D  E  I  N  G  S  D  E  F  D
ATCCGAGATCGATGAATCAGAGTGAGAAGAAAGTACTTAGTAGAACAGAGAGTAGAAGTC  360
 P  R  S  M  N  Q  S  E  K  K  V  L  S  R  T  E  S  R  S  L
TGTTTGAGTTCAAGTGTGTTCCTTTATATGGAGTGACTTCGATTTGTGGTAGACGACCAG  420
 F  E  F  K  C  V  P  L  Y  G  V  T  S  I  C  G  R  R  P  E
AGATGGAAGATTCTGTCTCAACGATTCCTAGATTCCTTCAAGTTTCTTCTAGTTCGTTGC  480
 M  E  D  S  V  S  T  I  P  R  F  L  Q  V  S  S  S  S  L  L
TTGATGGTCGAGTCACTAATGGATTTAATCCTCACTTGAGTGCTCATTTCTTTGGTGTTT  540
 D  G  R  V  T  N  G  F  N  P  H  L  S  A  H  F  F  G  V  Y
ACGATGGCCATGGCGGTTCTCAGGTAGCGAATTATTGTCGTGAGAGGATGCATCTGGCTT  600
 D  G  H  G  G  S  Q  V  A  N  Y  C  R  E  R  M  H  L  A  L
TGACGGAGGAGATAGTGAAGGAGAAACCGGAGTTTTGTGACGGTGACACGTGGCAAGAGA  660
 T  E  E  I  V  K  E  K  P  E  F  C  D  G  D  T  W  Q  E  K
AGTGGAAGAAGGCTTTGTTCAACTCTTTTATGAGAGTTGACTCGGAGATTGAAACTGTGG  720
 W  K  K  A  L  F  N  S  F  M  R  V  D  S  E  I  E  T  V  A
CTCATGCTCCGGAAACTGTTGGGTCTACCTCGGTGGTTGCGGTTGTCTTTCCGACTCACA  780
 H  A  P  E  T  V  G  S  T  S  V  V  A  V  V  F  P  T  H  I
TCTTTGTCGCGAATTGCGGCGACTCTAGGGCGGTTTTGTGTCGCGGCAAAACGCCACTCG  840
 F  V  A  N  C  G  D  S  R  A  V  L  C  R  G  K  T  P  L  A
CGTTGTCGGTTGATCACAAACCGGATAGGGATGATGAAGCGGCGAGGATAGAAGCTGCCG  900
 L  S  V  D  H  K  P  D  R  D  D  E  A  A  R  I  E  A  A  G
GTGGGAAAGTAATCCGGTGGAACGGGGCTCGTGTATTTGGTGTTCTCGCAATGTCAAGAT  960
 G  K  V  I  R  W  N  G  A  R  V  F  G  V  L  A  M  S  R  S
CCATTGGCGATAGATACCTTAAACCGTCAGTAATTCCGGATCCAGAAGTGACTTCAGTGC  1020
 I  G  D  R  Y  L  K  P  S  V  I  P  D  P  E  V  T  S  V  R
GGCGAGTAAAAGAAGATGATTGTCTCATCTTAGCAAGTGATGGTCTTTGGGATGTAATGA  1080
 R  V  K  E  D  D  C  L  I  L  A  S  D  G  L  W  D  V  M  T
CAAACGAAGAAGTGTGCGATTTGGCTCGGAAACGGATTTTACTATGGCATAAGAAGAACG  1140
 N  E  E  V  C  D  L  A  R  K  R  I  L  L  W  H  K  K  N  A
CGATGGCCGGAGAGGCTTTGCTTCCGGCGGAGAAAAGAGGAGAAGGAAAAGATCCTGCAG  1200
 M  A  G  E  A  L  L  P  A  E  K  R  G  E  G  K  D  P  A  A
CAATGTCCGCGGCAGAGTATTTGTCGAAGATGGCTTTGCAAAAAGGAAGCAAAGACAATA  1260
 M  S  A  A  E  Y  L  S  K  M  A  L  Q  K  G  S  K  D  N  I
TAAGTGTGGTAGTGGTTGATTTGAAGGGAATAAGGAAATTCAAGAGCAAATCCTTGAATt  1320
 S  V  V  V  V  D  L  K  G  I  R  K  F  K  S  K  S  L  N  *
gaaaaagaaggtttggaagaaaagtgaaaaaaaaagttttgatggtgggtaaaaattctc  1380
tttagtgaaaaaagaaagataaaacaacaggtaataattacattgtaatattaatttcct  1440
gcttaaatttgttatttactttctcaaaaa                                1470
```

B

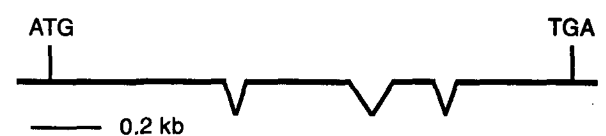

Figure 1. Structure of the *ABI2* cDNA and Gene.

(A) Nucleotide and deduced amino acid sequence of the *ABI2* cDNA (Arabidopsis ecotype Columbia). The coding sequence is shown in uppercase letters; the remainder of the nucleotides are shown in lowercase letters. At its 3′ end, the pcABI2 cDNA insert contains a poly(A) stretch of >100 nucleotides, only five of which are shown here. In the genomic sequence, an in-frame TGA stop codon is found 63 nucleotides 5′ to the first ATG translation initiation codon, suggesting that no coding sequences occur upstream from this designated start codon. The predicted amino acid sequence is shown directly below the nucleotide sequence. The asterisk indicates the stop codon. The *ABI2* coding region contains a silent nucleotide difference in the codon for Lys-192 between the Columbia (A at position 626 in the cDNA sequence) and Landsberg *erecta* (G at position 626) ecotypes. In addition, nucleotides 30 to 32 in the 5′ untranslated region of the Columbia cDNA are deleted in Landsberg *erecta*. The *abi2-1* mutation (Koornneef et al., 1984) is a G-to-A transition at nucleotide 553, which destroys the NcoI site extending from nucleotides 548 to 553 (underlined) and converts Gly-168 to Asp.

(B) Structure of the *ABI2* gene. Exons are denoted by thick lines, and the three introns are indicated by thin V-shaped lines and are located with respect to the cDNA sequence 3′ to nucleotides 612 (82 bp), 991 (146 bp), and 1243 (84 bp). The positions of the ATG and TGA codons delimiting the large open reading frame are indicated. The sequences reported in this article have been deposited in the EMBL database: accession numbers Y08965 (*ABI2* cDNA, ecotype Columbia) and Y08966 (*ABI2* gene, ecotype Landsberg *erecta*).

tested whether the *abi2-1* mutation could be located in the same stretch of nucleotides (which also contains a NcoI 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.

Fragments 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 NcoI. 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 NcoI site. The *abi2-1* mutation is a G-to-A transition at position 553 in the cDNA sequence and converts Gly-168 to Asp in the predicted ABI2 protein (Figure 1A). This was surprising because the *abi2-1* mutation, which originally was described as recessive (Koornneef et al., 1984; but see below), results in the same amino acid substitution and in the equivalent position in the phosphatase domain as that for the (semi)dominant *abi1-1* mutation (Gly-180 to Asp in *abi1-1*) (Figure 2).

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 (T_0 plants) were obtained from screening ~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 T_1 seed progeny (which segregated for the transgene) was markedly higher than that of the wild type. Like the *abi2-1* mutant, T_1 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

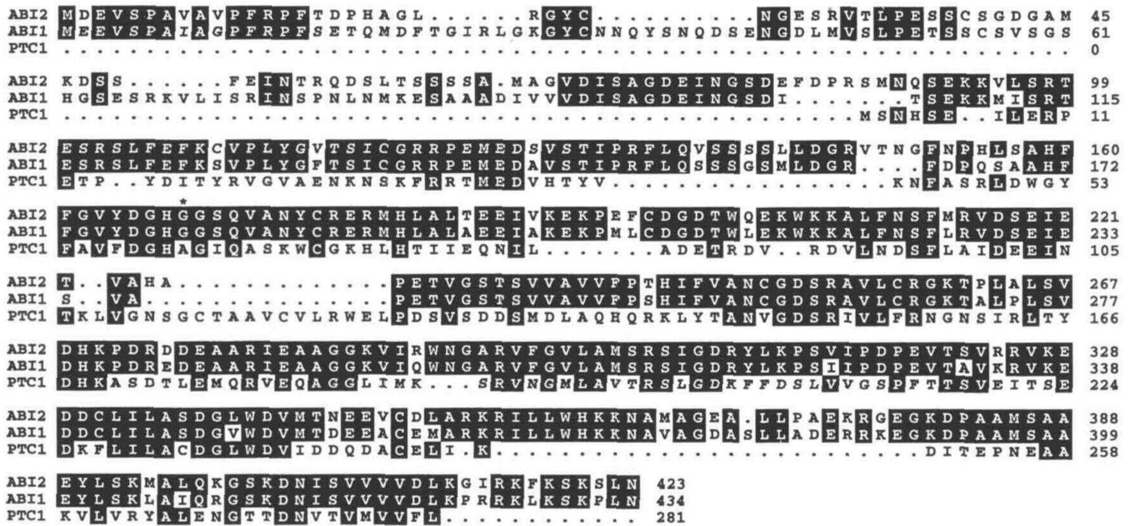

Figure 2. Comparison of the Predicted Amino Acid Sequence of the ABI2 Protein to ABI1 Protein and PTC1 PP2C.

Shown is alignment of the predicted sequences of ABI2, ABI1 (Leung et al., 1994; Meyer et al., 1994), and PTC1 PP2C from yeast (Maeda et al., 1993). Residues conserved between ABI2 and at least one of the ABI1 and PTC1 sequences are displayed as reverse print. Dots indicate gaps introduced to maximize alignments of conserved residues. The *abi2-1* mutation (Koornneef et al., 1984) converts Gly-168 of the ABI2 protein (indicated by the asterisk) to Asp. A similar substitution occurs in the ABI1 protein, with Gly-180 being converted to Asp (also as a result of G-to-A transition) in the *abi1-1* mutant (Leung et al., 1994; Meyer et al., 1994).

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 *abi2-1* 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 (T_0 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 *abi2-1* 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 T_1 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-

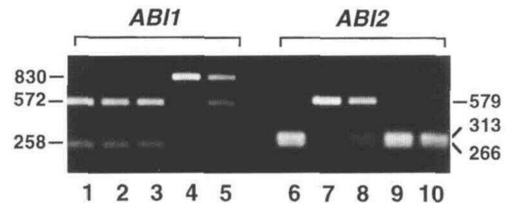

Figure 3. A PCR-Based Assay for Determining the Genotype of Plants at the *ABI1* and *ABI2* Loci.

DNA fragments encompassing the NcoI restriction sites in the *ABI1* (lanes 1 to 5) or *ABI2* (lanes 6 to 10) gene were amplified by PCR from genomic DNA from Landsberg *erecta* wild type (lanes 1 and 6), homozygous *abi2-1* mutant (lanes 2 and 7), heterozygous +/*abi2-1* (lanes 3 and 8), homozygous *abi1-1* mutant (lanes 4 and 9), and heterozygous +/*abi1-1* (lanes 5 and 10) plants. These amplification products were then digested with NcoI, and fragments were run on a 1.5% agarose gel and stained with ethidium bromide. Lengths of restriction fragments are indicated in base pairs. Whereas *abi1-1*, *abi2-1*, and wild-type plants are difficult to distinguish visually from each other in the absence of water stress, this molecular assay provides a convenient means to ascertain the genotype of individual plants used in phenotypic studies.

Table 1. Characteristics of Wild-Type Plants Transformed with the Mutant *abi2-1* Gene or cDNA

Genotype	Kanamycin Resistance[a] (%)	Seed Dormancy[b] (%)	Transpiration[c]	Germination on ABA[d] (%)
Wild type	0	5	17.23 ± 0.81	0
abi2-1 mutant	0	100	51.15 ± 5.80[e]	100
cosabi2 line A	70	39	14.77 ± 0.76	46
cosabi2 line B	95	70	16.09 ± 0.82	0
cosabi2 line C	98	42	13.88 ± 0.89	0
EN35S::*abi2* line 3	87	80	29.15 ± 0.67[e]	81
EN35S::*abi2* line 6	94	49	24.53 ± 0.85[e]	0

[a] Percentage of T_1 seeds that germinated on 50 mg/L kanamycin.
[b] Percentage of freshly harvested T_1 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_0 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_1 seeds that germinated after 3 days on 3 μM ABA.
[e] $P \leq 0.025$, two-sample unpaired Student's *t* test.

ing various phenotypes characteristic of the original *abi2-1* mutant. All transformants displayed reduced seed dormancy. The additional phenotypes (increased transpiration, resistance of seed germination to exogenous ABA) were observed only in a subset of the viable transgenic lines, suggesting that these responses may require a higher dosage of the mutant protein. Furthermore, six independent lines derived from transformation of wild-type plants with the cosmid cos27-1 that contains the wild-type *ABI2* gene (see above) displayed none of the above-mentioned mutant phenotypes (data not shown). Thus, taken together, our molecular and functional results indicate that the *ABI1* homolog is *ABI2.*

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 *ptc1*Δ 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 *ptc1*Δ (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 ^{32}P-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–to–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 *ptc1*Δ 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 μM 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,

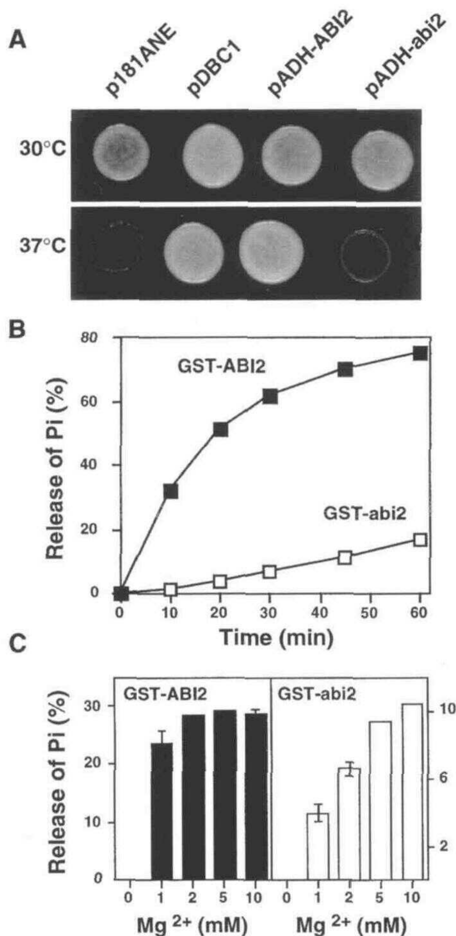

Figure 4. PP2C Activity of ABI2 and Impact of the *abi2-1* Mutation.

(A) Complementation studies of the *ptc1*Δ 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 ^{32}P-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 ^{32}Pi 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 ^{32}Pi 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+}.

the *ABI2* mRNA appeared to be more weakly expressed in leaves. Figure 6B shows that the *ABI1* and *ABI2* mRNAs were induced by sorbitol, an experimental treatment that mimicks conditions of low water potential (Zhu et al., 1993). The abundance of both transcripts was reduced in the ABA-deficient *aba1* mutant (Figure 6B), suggesting that endogenous ABA upregulates the expression of these mRNAs. Exogenous ABA indeed triggered accumulation of the *ABI1* and *ABI2* transcripts (Figure 6C). The mRNA levels were reduced in the *abi1-1* mutant and less severely in *abi2-1* (Figure 6C). This result suggests that enhancement of the *ABI* transcripts by ABA might involve *ABI1* and possibly *ABI2* as part of an inter- and autoregulated circuitry, either at the level of transcription or mRNA stability.

Heterozygous F_1 progeny were derived from reciprocal crosses between the wild type and either the *abi1-1* or *abi2-1* mutant. Both the *abi1-1* and *abi2-1* mutations were fully dominant over the corresponding wild-type allele in reducing ABA sensitivity of seed germination (Figure 5A), whereas they were semidominant in reducing ABA sensitivity of root growth (Figure 5B). Similar results were obtained regardless of whether the *abi* mutation was introduced from the paternal (Figures 5A and 5B) or maternal (data not shown) parent, indicating that *abi2-1* is zygotic.

DISCUSSION

Among the various ABA sensitivity mutations characterized so far in Arabidopsis (Koornneef et al., 1984; Finkelstein, 1994a; Cutler et al., 1996), only *abi1-1* and *abi2-1* 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 *abi1-1* and *abi2-1* 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 coincidental 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-

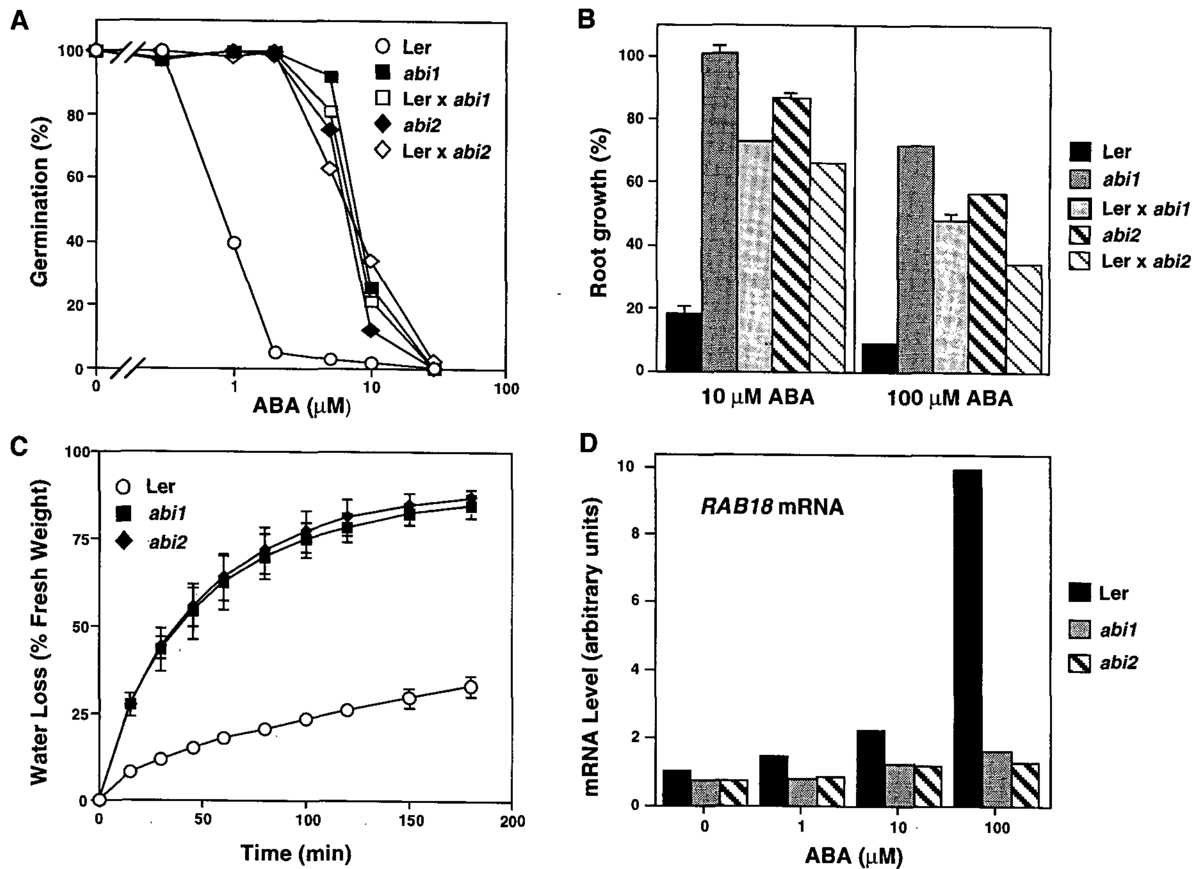

Figure 5. Phenotypic Comparison of the *abi1-1* and *abi2-1* Mutants.

ABA responses were analyzed for plant genotypes that were all in the Landsberg *erecta* background: wild type (L*er*), homozygous mutants *abi1-1* (*abi1*) and *abi2-1* (*abi2*), and heterozygous F_1 progeny from L*er* × *abi1* and L*er* × *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 cm^2) 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 (L*er*) 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 ABI2), 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 Mg^{2+} 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.

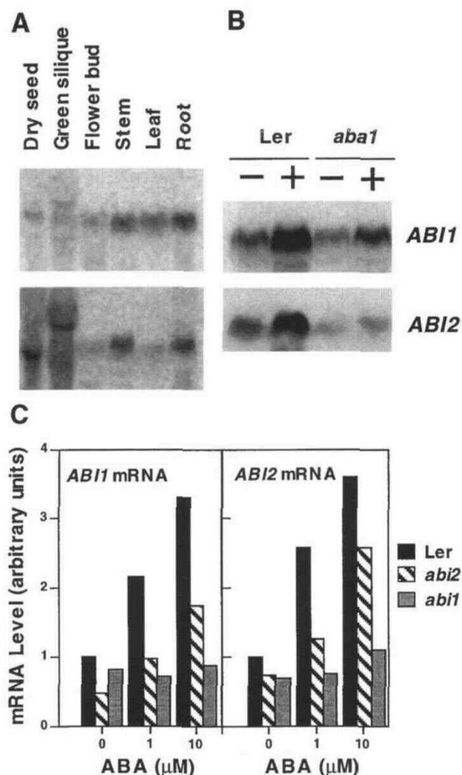

Figure 6. RNA Gel Blot Analyses of ABI1 and ABI2 Expression.

(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 *ABI* 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 (L*er*) and from the ABA biosynthetic mutant *aba1-1* (*aba1*) (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. *ABI* mRNA levels are expressed in arbitrary units, with 1 unit corresponding to the mRNA level in wild-type (L*er*) 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

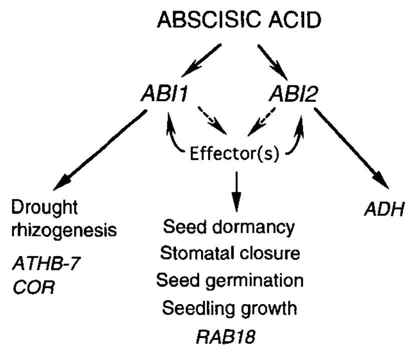

Figure 7. A Model for the Respective Roles of ABI1 and ABI2 in ABA Signaling.

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., 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.

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 transition have been isolated. It is possible that other potential dominant mutations may have escaped detection by the genetic screen, if, for example, they severely impair embryonic development or result in phenotypes other than resistance to ABA at the germination stage. Alternatively, the *abi1-1* and *abi2-1* mutations could be neomorphic (gain of function). Although this possibility cannot be formally excluded, we think it is less likely. First, the phosphatase activities of the mutant proteins are markedly reduced in heterologous expression systems, and thus, in molecular terms, the mutations correspond more appropriately to "loss of function." Second, the semidominant nature of the mutations with respect to root growth on ABA and transpiration suggests that the abi1-1 and abi2-1 proteins may still retain a sufficiently native conformation to compete with their wild-type counterparts in substrate binding. These results imply that the *abi1-1* and *abi2-1* mutations are rather more akin to "dominant negative." This situation is analogous to some catalytically inactive protein phosphatases (such as protein tyrosine phosphatases and calcineurin) whose corresponding mutations nonetheless behave genetically and/or molecularly as dominant (Sun et al., 1993; Herbst et al., 1996; Shibasaki et al., 1996).

Although a number of phenotypes between the *abi1-1* and *abi2-1* mutants are highly similar, the roles of their respective proteins in ABA signaling are unlikely to be completely redundant (Figure 7). Induction of the *RAB18* transcript by exogenous ABA in plantlets is similarly inhibited in the *abi1-1* and *abi2-1* mutants (Figure 5D; Gosti et al., 1995). Nevertheless, the ABA-dependent accumulation of this same transcript triggered by progressive drought in the roots of adult plants is severely inhibited in *abi1-1* but not in *abi2-1* (Gosti et al., 1995). Additional adaptive responses, such as root morphogenesis (drought rhizogenesis; Vartanian et al., 1994) as well as accumulations of the *AtDi21* (Gosti et al., 1995) and *ATHB-7* (Söderman et al., 1996) transcripts provoked by water stress, are affected in *abi1-1* but not in *abi2-1*. Conversely, inductions of the *Adh* transcript by water stress and by exogenous ABA are affected in *abi2-1* but not in *abi1-1* (de Bruxelles et al., 1996).

These dissimilarities hint at the existence of distinct upstream regulatory mechanisms, one of which may involve direct protein–protein interactions. For instance, the divergent N-terminal extensions may function as modules by binding to regulatory proteins that dictate cellular localization and substrate specificities of the ABI phosphatases (Hubbard and Cohen, 1993; Faux and Scott, 1996). Furthermore, different and overlapping combinations of such regulatory proteins coopted by ABI1 and ABI2 would present a versatile system highly responsive to subtle changes in environmental and cellular signals. It is clear that identifying regulatory proteins as well as physiological substrates of ABI1 and ABI2 would thus be a crucial step in understanding how common and different combinatorial protein interactions and their disruption in these mutants are implicated in the various facets of ABA signal transduction.

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 pcABI1-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 × SSC (1 × 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 CIC10B4 on chromosome 5, has been described by R. Schmidt (http://genome-www.stanford.edu/Arabidopsis/JIC-contigs/Chr5_YACcontigs.html). The *abi2-1* 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 dGTP and dATP. 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 XhoI site (incompletely filled in with dTTP and dCTP) of the cosmid pDCL04541 (Jones et al., 1992). The ligation mix was packaged using Gigapack II (Stratagene, La Jolla, CA) and used to infect the *Escherichia coli* DH5α. Wild-type cDNA clones were isolated from a library constructed in λgt10 by using poly(A)$^+$ RNA isolated from stems, leaves, and roots of in vitro–grown Arabidopsis plants (ecotype Columbia).

To isolate the mutant *abi2-1* gene, total genomic DNA was extracted (Leung et al., 1994) from Arabidopsis plants homozygous for both the *abi2-1* and the closely linked *yellow inflorescence* mutations, and size-fractionated DNA was then cloned in the cosmid pDCL04541 as described above. A cosmid containing the mutant *abi2-1* gene (cosabi2) was selected by screening the genomic library with the wild-type pcABI2 cDNA as probe.

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 *abi2-1* 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′-GCCATGACGGTTCTCAG-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 SmaI and cloned into the SmaI site of the binary plant transformation vector pDE1070 (Parcy et al., 1994). The construct was introduced into the *Agrobacterium tumefaciens* C58C1Rif(pGV2260) by electroporation (Wen-Jun and Forde, 1989). The cosabi2 cosmid containing the mutant *abi2-1* gene was introduced into the *E. coli* S17-1 (Simon et al., 1983) by electroporation and then mobilized into the Agrobacterium pGV3101 (pMP90RK) by bacterial conjugation (Koncz and Schell, 1986).

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 hr. 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 μM 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′-GATATCTCCGCCGGAGAT-3′ and 5′-CCATTCCACTGAATCACTTT-3′. The oligonucleotide primers used for amplification of the *ABI2* gene fragment were 5′-CATCATCTGCTATGGCAGG-3′ and 5′-CCGGAGCATGAGCCACAG-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.

PP2C Activities

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′-GTTGAATTCTAATGGACGAAGTTTC-3′ and 5′-GTTGAATTCCAATTAACCCTCACTAAAG-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 (*ptc1*Δ) (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 pGEX-2TK (Pharmacia Biotechnology) to generate in-frame fusion proteins with glutathione *S*-transferase (GST), resulting in GST–ABI2 and GST–abi2. Recombinant proteins were expressed in the *E. coli* DH5α and were affinity purified on glutathione Sepharose 4B resin (Bertauche et al., 1996). Phosphatase activity was determined by standard procedures, using ^{32}P-labeled casein as substrate (MacKintosh, 1993; Bertauche et al., 1996). Reactions were done in duplicate, and phosphatase activity was expressed as the percentage of ^{32}Pi released. Values for the GST–ABI2 and GST–abi2 proteins were subtracted for the amount of ^{32}Pi released in control samples containing native GST (always <1%).

Phenotypic and Molecular Analyses of the *abi1-1* and *abi2-1* Mutants

Landsberg *erecta* wild type, *abi1-1* (isolation number AII), and *abi2-1* (EII) (Koornneef et al., 1984) mother plants were grown simultaneously. Mature progeny seeds were harvested at the same time and used for subsequent phenotypic comparisons. Surface-sterilized seeds were placed on germination medium agar plates, chilled for 3 days at 4°C in darkness to break seed dormancy, and then incubated at 21°C with a 16-hr-light photoperiod (Parcy et al., 1994). For measurements of water loss, detached leaves were incubated abaxial face up in ambient laboratory conditions, and their fresh weight was measured at various time points. Water loss was expressed as the percentage of initial fresh weight upon excision. For measurements of *RAB18*, *ABI1*, and *ABI2* mRNA levels, total RNA was extracted and subjected to gel blot analysis under stringent conditions (Parcy et al., 1994). The *RAB18* cDNA probe has been previously described (Gosti et al., 1995). For *ABI1* and *ABI2* mRNA detection, gene-specific probes corresponding to the more divergent N-terminal domains of ABI1 (nucleotides 290 to 820 of pcABI1-c38) and ABI2 (nucleotides 1 to 459 of pcABI2) were used. Filters were then stripped and hybridized with an 18S rRNA probe. Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and for each RNA sample, signal with the *RAB18*, *ABI1*, or *ABI2* probe was standardized to that with the 18S rRNA probe.

ACKNOWLEDGMENTS

We thank David Bouchez and David Barford for communicating results before publication; Maarten Koornneef for Arabidopsis seeds; Haruo Saito for the plasmid pDBC1 and the yeast strain TM126; Wolfgang Frommer for the vector p181ANE; Clare Lister and Caroline Dean for the vector and map of pDCL04541; Andreas Bachmair for the λgt10 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. BIO4-CT96-0062).

Received December 6, 1996; accepted March 17, 1997.

REFERENCES

Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H., and Trewavas, A.J. (1994). Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. Plant Cell **6,** 1319–1328.

Anderson, B.E., Ward, J.M., and Schroeder, J.I. (1994). Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. Plant Physiol. **104,** 1177–1183.

Armstrong, F., Leung, J., Grabov, A., Brearley, J., Giraudat, J., and Blatt, M.R. (1995). Sensitivity to abscisic acid of guard cell K^+ channels is suppressed by *abi1-1*, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. Proc. Natl. Acad. Sci. USA **92,** 9520–9524.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.R. Acad. Sci. Ser. III Sci. Vie **316,** 1194–1199.

Bertauche, N., Leung, J., and Giraudat, J. (1996). Protein phosphatase activity of abscisic acid insensitive 1 (ABI1) protein from *Arabidopsis thaliana.* Eur. J. Biochem. **241,** 193–200.

Chandler, P.M., and Robertson, M. (1994). Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45,** 113–141.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58,** 453–508.

Creusot, F., Fouilloux, E., Dron, M., Lafeuriel, J., Picard, G., Billault, A., Le Paslier, D., Cohen, D., Chaboute, M.E., Durr, A., Fleck, J., Gigot, C., Camilleri, C., Bellini, C., Caboche, M., and Bouchez, D. (1995). The CIC library: A large insert YAC library for genome mapping in *Arabidopsis thaliana.* Plant J. **8,** 763–770.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996). A protein farnesyl transferase involved in

abscisic acid signal transduction in *Arabidopsis.* Science **273,** 1239–1241.

Das, A.K., Helps, N.R., Cohen, P.T.W., and Barford, D. (1996). Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 Å resolution. EMBO J. **15,** 6798–6809.

de Bruxelles, G.L., Peacock, W.J., Dennis, E.S., and Dolferus, R. (1996). Abscisic acid induces the alcohol dehydrogenase gene in *Arabidopsis.* Plant Physiol. **111,** 381–391.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12,** 387–395.

Faux, M.C., and Scott, J.D. (1996). More on target with protein phosphorylation: Conferring specificity by location. Trends Biochem. Sci. **21,** 312–315.

Finkelstein, R.R. (1994a). Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. Plant J. **5,** 765–771.

Finkelstein, R.R. (1994b). Maternal effects govern variable dominance of two abscisic acid response mutations in *Arabidopsis thaliana.* Plant Physiol. **105,** 1203–1208.

Finkelstein, R.R., and Somerville, C.R. (1990). Three classes of abscisic acid (ABA)–insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol. **94,** 1172–1179.

Gilmour, S.J., and Thomashow, M.F. (1991). Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana.* Plant Mol. Biol. **17,** 1233–1240.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F., and Goodman, H.M. (1992). Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell **4,** 1251–1261.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.-C., Bouvier-Durand, M., and Vartanian, N. (1994). Current advances in abscisic acid action and signaling. Plant Mol. Biol. **26,** 1557–1577.

Gosti, F., Bertauche, N., Vartanian, N., and Giraudat, J. (1995). Abscisic acid–dependent and –independent regulation of gene expression by progressive drought in *Arabidopsis thaliana.* Mol. Gen. Genet. **246,** 10–18.

Herbst, R., Carroll, R.M., Allard, J.D., Schilling, J., Raabe, T., and Simon, M.A. (1996). *Daughter of sevenless* is a substrate of the phosphotyrosine phosphatase *corkscrew* and functions during *sevenless* signaling. Cell **85,** 899–909.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science **269,** 1712–1714.

Hubbard, M.J., and Cohen, P. (1993). On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem. Sci. **18,** 172–177.

Jones, J.D.G., Schlumukov, L., Carland, F., English, J., Scofield, S.R., Bishop, B.J., and Harrison, K. (1992). Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Res. **1,** 285–297.

Koncz, C., and Schell, J. (1986). The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. **204,** 383–396.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403–410.

Koornneef, M., Jorna, M.L., Brinkhorst-Van der Swan, D.L.C., and Karssen, C.M. (1982). The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **61,** 385–393.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The isolation and characterization of abscisic acid–insensitive mutants of *Arabidopsis thaliana.* Physiol. Plant. **61,** 377–383.

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994). *Arabidopsis* ABA-response gene *ABI1*: Features of a calcium-modulated protein phosphatase. Science **264,** 1448–1452.

MacKintosh, C. (1993). Assay and purification of protein (serine/threonine) phosphatases. In Protein Phosphorylation: A Practical Approach, D.G. Hardie, ed (Oxford, UK: Oxford University Press), pp. 197–230.

Maeda, T., Tsai, A.Y.M., and Saito, H. (1993). Mutations in a protein tyrosine phosphatase gene (*PTP2*) and a protein serine/threonine phosphatase gene (*PTC1*) cause a synthetic growth defect in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13,** 5408–5417.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., and Vasil, I.K. (1991). The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell **66,** 895–905.

Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana.* Science **264,** 1452–1455.

Nambara, E., Keith, K., McCourt, P., and Naito, S. (1995). A regulatory role for the *ABI3* gene in the establishment of embryo maturation in *Arabidopsis thaliana.* Development **121,** 629–636.

Ooms, J.J.J., Léon-Kloosterziel, K.M., Bartels, D., Koornneef, M., and Karssen, C.M. (1993). Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana.* A comparative study using abscisic acid–insensitive *abi3* mutants. Plant Physiol. **102,** 1185–1191.

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during Arabidopsis seed development: Roles of the *ABI3* locus and of endogenous abscisic acid. Plant Cell **6,** 1567–1582.

Schnall, J.A., and Quatrano, R.S. (1992). Abscisic acid elicits the water-stress response in root hairs of *Arabidopsis thaliana.* Plant Physiol. **100,** 216–218.

Schwartz, A., Wu, W.-H., Tucker, E.B., and Assmann, S.M. (1994). Inhibition of inward K^+ channels and stomatal response by abscisic acid: An intracellular locus of phytohormone action. Proc. Natl. Acad. Sci. USA **91,** 4019–4023.

Shibasaki, F., Price, E.R., Milan, D., and McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature **382,** 370–373.

Simon, R., Preifer, U., and Pühler, A. (1983). A broad host range mobilization system for in vitro genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1,** 784–791.

Söderman, E., Mattson, J., and Engström, P. (1996). The *Arabidopsis* homeobox gene *ATHB-7* is induced by water deficit and by abscisic acid. Plant J. **10,** 375–381.

Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell **75,** 487–493.

Vartanian, N., Marcotte, L., and Giraudat, J. (1994). Drought rhizogenesis in *Arabidopsis thaliana*. Differential responses of hormonal mutants. Plant Physiol. **104,** 761–767.

Ward, J.M., Pei, Z.-M., and Schroeder, J.I. (1995). Roles of ion channels in initiation of signal transduction in higher plants. Plant Cell **7,** 833–844.

Wen-Jun, S., and Forde, B.G. (1989). Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucleic Acids Res. **17,** 8385.

Zhu, B., Chen, T.H.H., and Paul, H.L. (1993). Expression of an ABA-responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol. Biol. **21,** 729–735.