μg/ml). Except for dormancy assays, the plates were first placed for 4 days at 4°C in the dark. They were then incubated in a growth chamber at 24°C with a 16-hour light photoperiod.
22. Deletion derivatives were generated by partial digestion with Bam HI or Bam HI and Sma I in combination, religated, and transformed into *Escherichia coli*. Seventy-five Tn5 insertion mutants were generated [P. Putnoky *et al.*, *J. Bacteriol.* **172**, 5450 (1990)] and mapped in the ΔE insert; seven of these mutants were subsequently selected to confirm the identity of the *abi1-1* locus by functional assay.
23. J. Leung and J. Giraudat, unpublished results.
24. The pcABI1-C38 clone originates from a cDNA library constructed from *Arabidopsis* leaves (Col wild type). The full insert and isolated restriction fragments were subcloned into pBluescript KS+ (Stratagene) for sequence determination. Six overlapping fragments encompassing the transcribed region of the *ABI1* gene were amplified by PCR (with the use of specific primers) from Lan wild-type genomic DNA and from the *abi1-1* mutant DNA insert of λE4-1R2. The amplified fragments were subcloned into pBluescript KS+ for sequence determination. Nucleotide differences were verified from independently generated PCR products. Double-stranded plasmid DNA was sequenced on an Applied Biosystems (Foster City, CA) automated DNA sequencer (model 373A) with the use of dye primers as recommended by the manufacturer. Sequence analyses were done with programs of the Wisconsin GCG software package (*38*).
25. Database searches were performed at the U.S. National Center for Biotechnology Information with the use of the BLAST Network Service [S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)].
26. N. D. Moncrief, R. H. Kretsinger, M. Goodman, *J. Mol. Evol.* **30**, 522 (1990).
27. A. Bairoch, *Nucleic Acids Res.* **19**, 2241 (1991).
28. J. Garnier, D. Osguthorpe, B. Robson, *J. Mol. Biol.* **120**, 97 (1978).
29. S. Tamura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1796 (1989).
30. T. Maeda, A. Y. M. Tsai, H. Saito, *Mol. Cell. Biol.* **13**, 5408 (1993).
31. P. Cohen, *Annu. Rev. Biochem.* **58**, 453 (1989).
32. M. R. McAinsh, C. Brownlee, M. Sarsag, A. A. R. Webb, A. M. Hetherington, in *Abscisic Acid: Physiology and Biochemistry*, W. J. Davies and H. G. Jones, Eds. (Bios Scientific, Oxford, 1991), pp. 137–152; S. Gilroy, M. D. Fricker, N. D. Read, A. J. Trewavas, in *Progress in Plant Growth Regulation*, C. M. Karssen, L. C. van Loon, D. Vreugdenhil, Eds. (Kluwer Academic, Dordrecht, Netherlands, 1992), pp. 105–115.
33. M. Bouvier-Durand, M. Real, D. Côme, *Plant Physiol. Biochem.* **27**, 511 (1989); M. Levi, P. Brusa, D. Chiatante, E. Sparvoli, *In Vitro Cell. Dev. Biol.* **29P**, 47 (1993).
34. T. Jacobs, *Dev. Biol.* **153**, 1 (1992); C. Staiger and J. Doonan, *Curr. Opin. Cell Biol.* **5**, 226 (1993).
35. Y. Lee and S. M. Assmann, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2127 (1991); S. Luan, W. Li, F. Rusnak, S. M. Assmann, S. L. Schreiber, *ibid.* **90**, 2202 (1993).
36. Three independent C24 transgenic lines homozygous for the λE4-1R2 transgene were tested on nine concentrations of ABA ranging from 0 to 10 μM. Concentrations of 0.2 μM ABA inhibited root elongation (scored after 4 days) in 50% of the C24 wild-type seeds and of 0.5 μM ABA in 100% of the C24 wild-type seeds. In the transgenic lines, up to 3 μM ABA had no detectable effect, whereas 8 to 10 μM ABA was required to inhibit root development in 50% of the seeds. Freshly harvested mature seeds of these transgenic lines also displayed reduced dormancy because, in the absence of added ABA, 100% of the transgenic seeds germinated within 2 days, whereas only 5% of the C24 wild-type seeds had germinated after 2 days and 25% of the seeds had germinated after 7 days.
37. J. Leung, D. A. R. Sinclair, S. Hayashi, G. M. Tener, T. A. Grigliatti, *J. Mol. Biol.* **219**, 175 (1991).
38. J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
39. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
40. We thank H. M. Goodman for support during the early phase of this work; B. Hauge, H. M. Goodman, and E. M. Meyerowitz for RFLP probes; S. Brown, B. Labidi, and J.-R. Prat for advice on microscopy; T. Peeters and M. Koornneef for seed stocks and unpublished genetic mapping data; M.-O. Fauvarque for initial RFLP analysis; C. Somerville and E. Ward for YAC banks; A. Bent, D. Dahlbeck, T. Tai, B. Staskawicz, F. Katagiri, M. Mindrinos, F. Ausubel, A. Bachmair, and M. Kreis for cDNA libraries and purified clones; F. Parcy and C. Valon for RNA samples and blots; B. Hoffmann, G. Petrovics, and E. Kondorosi for materials and protocols on Tn5 mutagenesis; Plant Genetics Systems for the T-DNA vector pDE1000; R. Drouen for gardening work; and J. Guern, B. Gronenborn, S. Brown, U. Bonas, and J. Laufs for comments on the manuscript. Supported by the Centre National de la Recherche Scientifique, the European Economic Community BRIDGE program (BIOT-CT90-0207), and the Ministère de la Recherche et de la Technologie (91.T.0441).

3 February 1994; accepted 26 April 1994

A Protein Phosphatase 2C Involved in ABA Signal Transduction in *Arabidopsis thaliana*

Knut Meyer, Martin P. Leube, Erwin Grill*

The plant hormone abscisic acid (ABA) mediates various responses such as stomatal closure, the maintenance of seed dormancy, and the inhibition of plant growth. All three responses are affected in the ABA-insensitive mutant *abi1* of *Arabidopsis thaliana*, suggesting that an early step in the signaling of ABA is controlled by the *ABI1* locus. The *ABI1* gene was cloned by chromosome walking, and a missense mutation was identified in the structural gene of the *abi1* mutant. The *ABI1* gene encodes a protein with high similarity to protein serine or threonine phosphatases of type 2C with the novel feature of a putative Ca^{2+} binding site. Thus, the control of the phosphorylation state of cell signaling components by the *ABI1* product could mediate pleiotropic hormone responses.

The sesquiterpenoid plant hormone ABA is involved in many aspects of growth and development such as embryo development, seed dormancy, and adaptation responses toward low water potentials (*1*). Under conditions of water shortage, ABA induces the closure of leaf stomata and the formation of specific proteins involved in the desiccation response. The amount of ABA increases during water stress, and high concentrations of ABA inhibit plant growth.

Abscisic acid affects the expression of several genes involved in seed maturation and the desiccation response (*2*). The gene expression is modulated by ABA through a *cis*-acting regulatory element (*3*), for which a DNA binding factor has been identified (*4*). In maize and *Arabidopsis*, two structurally similar DNA binding proteins are involved in the control of seed maturation and dormancy (*5*). Individual components that act in the signaling cascade of ABA in the stomatal response are just beginning to emerge. Inositoltrisphosphate and Ca^{2+} can evoke stomatal closure (*6*), a process that requires the concerted regulation of at least four different ion channels, including channels for Ca^{2+} and anions as well as inward- and outward-rectifying K^+ channels (*7*). High-affinity binding sites for ABA at the cytoplasmic membrane of stomatal guard cells have been identified (*8*) but not further characterized.

The loci *ABI1* and *ABI2* of *Arabidopsis* are possible candidates for the encoding of an ABA perception site or early steps in its signal transduction (*9*). Mutants of both loci were found to be ABA-insensitive and revealed pleiotropic alterations in their responses toward ABA, whereas endogenous ABA levels and the catabolism of ABA were not affected. The dominant *ABI1* mutation is characterized by causing a wilty phenotype as well as ABA-insensitive seed germination and growth.

We are interested in elucidating the individual steps that lead to the control of plant growth by ABA. In this report, we describe the identification of the *Arabidopsis ABI1* gene. The gene encodes a product that has sequence homology with the class of protein serine or threonine phosphatase 2C. The ABI1 gene product has the additional features of a putative adenosine triphosphate (ATP) or guanosine triphosphate (GTP) binding site and an amino-terminal extension with a possible Ca^{2+} binding site.

The *ABI1* locus has been mapped to the chromosome 4 of *Arabidopsis* between the genetic markers *cer2* and *ap2* at a position

Institute of Plant Sciences, Swiss Federal Institute of Technology, Universitätsstrasse 2, 8092 Zürich, Switzerland.

*To whom correspondence should be addressed.

3.0 ± 1.5 centimorgans (cM) distal from *cer2* and 15.9 ± 2.8 cM proximal from *ap2* (*10*). We used molecular markers that reveal restriction fragment length polymorphism (RFLP) between different ecotypes of *Arabidopsis* to map the locus more closely (*11*). The marker λAt600 cosegregated with the *ABI1* locus. Only one recombination event among 1032 F2 chromosomes analyzed was identified between the locus and the marker λAt600. Using the molecular marker as a probe, we screened yeast artificial chromosome (YAC) libraries of *Arabidopsis* (*12*) and identified several corresponding YAC clones (*13*). End probes of these YACs, generated by inverse polymerase chain reaction (PCR) and by plasmid rescue of the left vector insert junctions (*14*), were used to extend the contiguous chromosomal fragments by the rescreening of the genomic libraries. Finally, a contig of overlapping YACs was established that extends about 260 kb distal and 240 kb proximal to the starting marker on chromosome 4 (*15*).

The position of the *ABI1* locus was refined by the localization of the chromosomal break points of previously identified recombinant chromosomes relative to new RFLP markers that were identified among subcloned fragments of the YAC contig. Thus, the location of the *ABI1* gene could be confined to a chromosomal fragment of 141 kb bordered by the markers λAt600 and cosIC9 (Fig. 1A). This chromosomal segment was cloned in three contiguous YACs, derived from DNA of the *Arabidopsis abi1* mutant (Fig. 1A). Subsequently, YAC DNA was isolated on a preparative scale by pulse field gel electrophoresis and subcloned as Hind III fragments into a binary cosmid vector in *Escherichia coli* (*16*). As a prelude to identify the dominant *abi1* gene by its ability to confer the mutant phenotype, the cloned fragments in the size range of 12 to 25 kb were assembled into a single contig encompassing 145 kb and including the *abi1* locus (*16*). Before the transformation of *Arabidopsis*, the cosmid clones were electroporated into *Agrobacterium tumefaciens* (*17*), and the integrity of the cloned *Arabidopsis* DNA fragments was examined by Hind III restriction digestion to test for instabilities such as those observed with other cosmid clones in *A. tumefaciens* (*18*). We did not observe any indication of instability; restriction patterns of cosmids propagated in *E. coli* and *A. tumefaciens* were identical, as were the patterns obtained by DNA blot analyses of *Arabidopsis* DNA with selected cosmids as probes (*13*).

A total of 18 different genomic fragments were mobilized from *Agrobacterium* into *Arabidopsis* root explants (ecotype RLD) along with two selectable markers, a kanamycin resistance gene and a β-glucuronidase reporter gene, which border the transfer DNA (T-DNA) (*15*). Transgenic plants (R_0 plants) were regenerated (*19*), and seeds of the individual transgenes (R_1 seeds) were tested for ABA insensitivity by

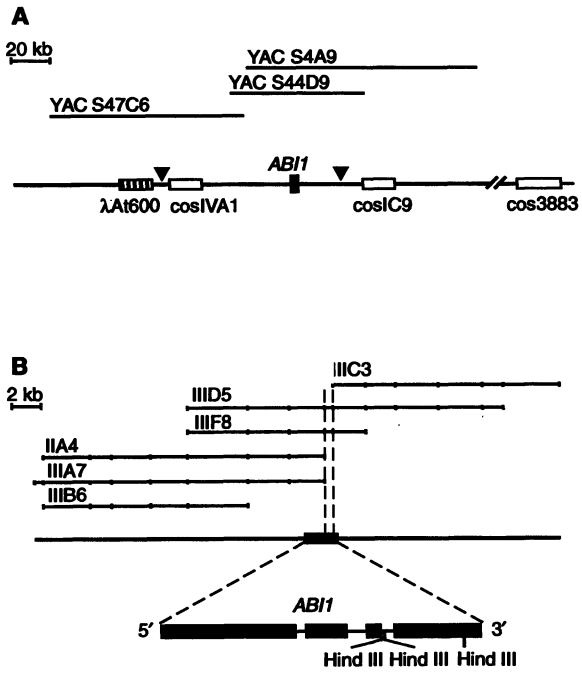

Fig. 1. Physical map of the *ABI1* locus relative to the chromosome walk contig. (**A**) The hatched bar signifies the starting point of the walk (λAt600) to the *ABI1* locus, indicated as a solid bar. Open bars represent other polymorphic DNA fragments used to localize recombination breakpoints. Markers cosIC9 and cosIVA1 are subcloned fragments of YAC S4A9 and YAC S47C6, respectively, which were isolated from a genomic library of the *abi1* mutant. Inverted triangles indicate the two closest breakpoints proximal and distal to *ABI1*. The single breakpoint detected between the *ABI1* locus and cosIC9 represents one out of 60 recombination events identified between the locus and the marker cos3883. The genetic distance between markers λAt600 and cos3883 is 11.3 cM. (**B**) Subcloned fragments of YAC S4A9 shown as solid lines on which Hind III restriction sites are indicated by ticks. The two fragments capable of conferring ABA insensitivity, IIID5 and IIIF8, contain a 0.6-kb fragment indicated by hatched vertical lines that is missing on other fragments of the contig. The position of the *ABI1* exons corresponding to a 2.0-kb cDNA clone is marked by a solid bar on the schematic genomic region and is magnified for better visibility of introns (shown as thin lines), including three Hind III restriction sites.

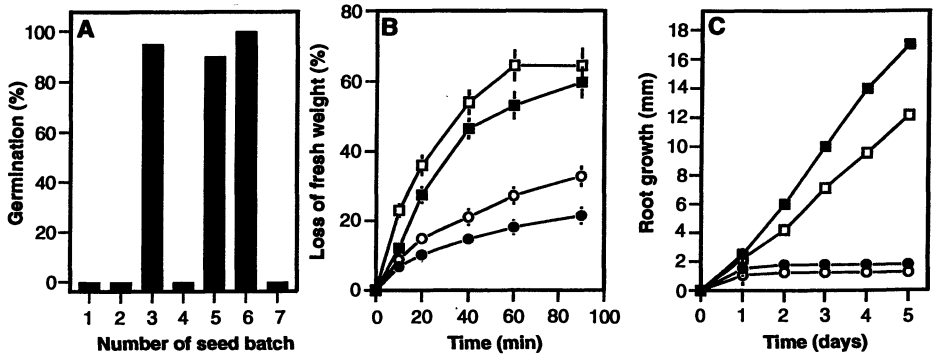

Fig. 2. Analyses of ABA-mediated responses of *Arabidopsis*. (**A**) The rate of seed germination in the presence of 3 μM (*R,S*)-*cis,trans*-ABA (Sigma), determined from 50 seeds of Landsberg *erecta* (*1*), RLD (*2*), and *abi1* mutant, genotype Landsberg *erecta* (*3*), after 5 days. In addition, the germination rate was assessed in R_1 seeds of RLD regenerates transformed with the T-DNA of the binary cosmid clone IIA4 (*4*) (32 seeds tested), IIIF8 (*5*) (36 tested), IIID5 (*6*) (42 tested), and IIIC3 (*7*) (30 tested). The analysis was performed as mentioned in (*9*). Germination without ABA was at least 95%. (**B**) The desiccation of excised leaves at room temperature, monitored as a function of time. Four leaves at approximately the same developmental stage and size from single 3-week-old plants of Landsberg *erecta* (open circles), RLD (filled circles), *abi1* mutant (open squares), and R_1 plants of RLD::IIID5 (filled squares) were analyzed by weighing (*9*). The assay was performed with three single plants each. The SD is indicated by vertical lines. (**C**) The growth of roots in the presence of 20 μM ABA, determined after transfer of 4-day-old seedlings of Landsberg *erecta*, RLD, *abi1* mutant, and R_1 plants of RLD::IIID5 onto ABA-containing medium [(symbols are as in (B)]. The results represent the average growth of 12 seedlings each, which were analyzed as mentioned in (*33*) with the modification that the medium contained only ABA as a plant hormone. The SD is indicated by vertical lines. The root growth of seedlings transferred onto control medium without ABA was 18.9 ± 1.2, 16.7 ± 0.9, 14.7 ± 1.0, and 16.6 ± 0.8 mm for Landsberg *erecta*, RLD, *abi1* mutant, and R_1 plants of RLD::IIID5, respectively, for 5 days.

their ability to germinate in the presence of 3 μM ABA, a concentration that is completely inhibitory to wild-type seeds under the conditions tested. Seeds that contain an additional single copy of the *abi1* mutant gene germinate. Two genomic fragments (Fig. 1B) of the cosmid contig, IIID5 and IIIF8, could confer an ABA-insensitive phenotype, in contrast to the fragments IIIA7, IIA4, and IIIC3. The independent R_1 seed batches of the cointegrates RLD::IIID5 (42 and 15 R_1 seeds, respectively) and RLD::IIIF8 (36 and 43 R_1 seeds, respectively) germinated with a frequency of 65 to 100% (42 and 13 seeds, and 32 and 28 seeds, respectively) in the presence of ABA (Fig. 2A). The nonsegregating mutant phenotype among the progeny of one RLD::IIID5 transgenic plant indicated that the original transformed plant had integrated two or more genetically unlinked copies of the mutant gene. In support of this view, 29 seedlings that were tested for the kanamycin marker were all resistant to the antibiotic. This R_1 progeny was also examined for ABA sensitivity to both the stomatal response (Fig. 2B) and root growth (Fig. 2C). All siblings of the transgenic plant examined revealed the ABA-insensitive phenotype of the *abi1* mutant. Seed material (at least 25 seeds examined each) of two, four, and six independent transgenic plants containing the fragments IIIA7, IIA4, and IIIC3, respectively, all showed ABA sensitivity typical of the wild type. The seed material was obtained from R_0 plants that expressed both kanamycin resistance and β-glucuronidase as an indication of the complete transfer and integration of the T-DNA into the plant genome. The cosmid clones used to produce these transgenic plants lacked a 0.6-kb fragment present on the two clones conferring ABA insensitivity (Fig. 1B), indicating that the *ABI1* gene is entirely or, more likely, partially located on this fragment.

Therefore, we screened a complementary DNA (cDNA) library of *Arabidopsis* (*20*) using this 0.6-kb fragment and the two flanking Hind III fragments (Fig. 1B) as a probe and identified seven corresponding cDNA clones among 320,000. All clones represented transcripts of a single gene. The clone λ*abi1-7* containing a 2.0-kb cDNA insert was used to localize the position of the gene exons on the physical map of the cosmid contig in a DNA blot analysis. Indeed, part of the cloned cDNA was found to be homologous to the 0.6-kb fragment (Fig. 1B). We conclude from these results that the *ABI1* gene was identified. In an RNA blot probed by the cDNA, a single transcript of approximately 2.1 kb was detected. No apparent difference in size was detected in RNA of wild-type, *abi1* mutant, or the allelic mutant *abi1-3* (*21*). Sequence analysis of the cDNA clone [1963 base pairs (bp)] revealed an open reading frame of 1302 bp from the presumed start codon, 400 bp of sequence preceding the start codon, and 246 bp after the translation stop codon as well as a polyadenylated [poly(A)$^+$] tail of 12 bases (*22*). The protein product is expected to have 434 amino acids. Comparison of the cDNA with the genomic sequence (*22*) indicates that the gene has three introns.

```
          1                                                                  
ABI1      MEEVSPAIAGPFRPPSETQMDFTGIRLGKGYCNNQYSNQDSENGDLMVSLPETSSCSVSGSHGSESRKVLISRINSPNLNMKESAAADIVVVDISAGDEING

          103                                                    ...DGHDG...
ABI1        SDVTSEKKMISRTESRSLFEFKSVPLYGFTSICGRRPEMEDAVSTIPRFLQSSSGSMLDGRFDPQSAAHFFGVYDGHGGSQVANYCRERMHLALAEEIAK
PTC1              MSNHSEILERPTPYDITYRVGVAENKNSKFRRTMEDVHTYVKNF---------ASRLDWG----YFAVFDGHAGIQASKWCGKHLHTIIEQNILA
RPP2C         MGAFLDKPKMEKHNAQGQGNGLRYGLSSMQGWRVEMEDAHTAVIGL--------PSGLETWS----FFAVYDGHAGSQVAKYCCEHLLDHITNN-QD
LPP2C         MGIPLPKPVMTQLQERYGNAIFRCGSNCVNGYRETMEDAHLTYLT------------DSWG----FFGVFDGHVNDQCSQYL----------ERAW

          203                                                                
ABI1        EKPMLCDGDTWLEKWKKALFNSFLRVDSEI-----ESVAPETVGSTSVVAVV----------------FPSHIFVANCGDSRAVLCRGKTALPLSVDHK
PTC1        DETRDV---------RDVLNDSFLAIDEEI-----NTKLVGNSGCTAAVCVLRWELPDSVSDDSMDLAQHQRKLYTANVGDSRIVLFRNGNSIRLYTDHK
RPP2C       PKGSAGAPSVENV--KNGIRTGFLEIDEHMRVMSEKKHGADRGGSTAVGVLI----------------SPQHTYPI--KCGDSRGLLCRNRKVHFFTQDHK
LPP2C       RSAIEKESIPMTD---ERMKELAIRIDQEWM----DSGREGGSTGTFFVALK----------------EGNKVHLQVGNVGDSRVVACIDGVCVPLTEDHK

          281                                                                
ABI1        PDREDEAARIEAAGGKVIQWNGARVFGVLAMSRSIGDRYLKPSIIPDPEVTAVKRVKE----------DDCLILASDGVWDV-MTDEEACEMARKRILLW
PTC1        ASDTLEMQRVEQAGGLIMK---SRVNGMLAVTRSLGDKFFDSLVVGSPFTTSVEITSE----------DKFLILACDGLWDV-IDDQDACELIK------
RPP2C       PSNPLEKERIQNAGGSVMI---QRVNGSLAVSRALGDFDYKCVHGKGPTEQLVSPEPEVHDIERSEEDDQFIILACDGIWDV-MGNEELCDFVRSRL---
LPP2C       PNNEGERQRIENCAGRVEN---NRVDGSLAVSRAFGDREYKLGSG-SQLEQKVIALADVQHKDFTFDSNDFVLLCCDGVFEGNFPNEEVVAYVKQQL---

          371                                                                
ABI1        HKKNAVAGDASLLADERRKEGKDPAAMSAAEYLSKLAIQRGSKDNISVVVVDLKPRRKLKSKPLN
PTC1        ------------------DITEPNE--AAKVLVRYALENGTTDNVTVMVVFL
RPP2C       ------------------EVTD-DLEKVCNEVVDTCLYKGSRDNMSVILICFPNAPKVSAEAVKKEAELDKYLENRVEEIIKKQGEGVPD.....
LPP2C       ------------------EICN-DLAEVAGRVCEEAIERGSRDNISCMIVQFKDGSDYAAEPHTTVVPGPFSAPRNSGFRKAYESMADKG.....
```

Fig. 3. Comparison between the predicted amino acid sequences of the *ABI1* gene product and of the protein serine or threonine phosphatases of *Saccharomyces cerevisiae PTC1* (*26*), rat RPP2C (*25*), and *Leishmania* LPP2C (*27*). Amino acids identically conserved in three out of four aligned sequences are printed in boldface. The putative binding sites for Ca^{2+}, DISAGDEINGSDV (*34*), and for ATP or GTP as a P-loop structure (*35*), AVLCRGKT, are marked by overstriking lines. The glycine substitution by aspartic acid in the *abi1* mutant gene product that is flanked by conserved residues (DGH and G) is aligned above the corresponding WT residues. The primary structures were aligned with the PILEUP program (*23*) and are shown in the single-letter code. The dots indicate a continuing amino acid sequence, and dashes mark gaps introduced by the alignment of the protein sequences.

The predicted protein sequence is similar to that of protein phosphatases 2C (Fig. 3) (*23*). Indeed, the ABI1 gene product heterologously expressed in *E. coli* exhibits Mg^{2+}-dependent protein phosphatase activity (*24*). The degree of homology in the carboxyl-terminal domain (residues 100 to 434) of the *ABI1* gene product (35% identity and 57% similarity to a mammalian protein phosphatase 2C, respectively) is as high as the structural homology among the protein phosphatases 2C from rat (*25*), yeast (*26*), and *Leishmania* (*27*). Among the characterized phosphatases of this type, only the *ABI1* product has the feature of an amino-terminal extension of approximately 100 amino acids with the conserved structural motif of a Ca^{2+} binding site (*23*) at residues 93 to 105 (Fig. 3). Present in the amino-terminal domain is the sequence RLGK-$[X]_8$-N-$[X]_{10}$-SLPE (*28*) (residues 26 to 52), which is identically preserved in the Ca^{2+} binding subunit of calcineurin from mammals and *Drosophila* (*23*). In addition, a putative ATP or GTP binding site AVLCRGKT (residues 264 to 271), which fits within the consensus motif A- or G-$[X]_4$-GK-S or -T (*23*), is present in the deduced primary structure of the *ABI1* product. The divergence from wild type occurs in the structural part of the *abi1* mutant gene (*22*), where a nucleotide base transition from guanine to adenine changes the DNA sequence GGC to GAC, thus causing the wild-type glycine^{180} residue to be replaced with aspartic acid (Fig. 3). The dominant nature of the mutation could be due to a constitutively active *abi1* gene product; for instance, the ABA signal could lead to the inactivation of the phosphatase activity in the wild-type but not in the mutant form or be due to a dominant negative mutation, as exemplified by complex poisoning. Even a mutation that causes an alteration of the substrate specificity of the gene product could be responsible for the dominant phenotype. The biochemical analysis of wild-type and mutant forms is required to help clarify this point.

Protein phosphatases of the type 2C are considered to be Mg^{2+}-dependent serine or threonine phosphatases localized in the cytoplasm (*29*). Protein serine or threonine phosphatases are also involved in signaling processes, such as calcineurin (type 2B) in T cell activation (*30*). A calcineurin-like protein phosphatase has been implicated in the regulation of stomatal aperture by the mediation of Ca^{2+}-induced inactivation of potassium channels (*31*), a process also elicited by ABA (*7*). The *ABI1* gene product is conceivably a candidate for this calcineurin-like protein in guard cells because it regulates stomatal aperture and has some, if limited, structural homology to calcineurin B. However, the action of the *ABI1* protein is not restricted to the control of stomatal opening. The *ABI1* protein also exerts control of germination and growth, its function in this respect resembling that of protein serine or threonine phosphatases

that has been identified in yeast as playing a crucial role in the control of growth (*26, 32*).

REFERENCES AND NOTES

1. W. J. Davies and H. G. Jones, *Abscisic Acid* (Bios Scientific, Oxford, 1991).
2. K. Skriver and J. Mundy, *Plant Cell* **2**, 503 (1990).
3. W. R. Marcotte Jr., S. H. Russell, R. S. Quatrano, *ibid.* **1**, 969 (1989); J. Mundy, K. Yamaguchi-Shinozaki, N.-H. Chua, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1406 (1990).
4. M. J. Guiltinan, W. R. Marcotte Jr., R. S. Quatrano, *Science* **250**, 267 (1990); K. Oeda, J. Salinas, N.-H. Chua, *EMBO J.* **10**, 1793 (1991).
5. D. R. McCarty *et al.*, *Cell* **66**, 895 (1991); J. Giraudat *et al.*, *Plant Cell* **4**, 1251 (1992).
6. M. R. Blatt, G. Thiel, D. R. Trentham, *Nature* **346**, 766 (1990); M. R. McAinsh, C. Brownlee, A. M. Hetherington, *ibid.* **343**, 186 (1990); S. Gilroy, N. D. Read, A. J. Trewavas, *ibid.* **346**, 769 (1990); J. I. Schroeder and S. Hagiwara, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9305 (1990); H. R. Irving, C. A. Gehring, R. W. Parish, *ibid.* **89**, 1790 (1992).
7. M. R. Blatt and G. Thiel, *Annu. Rev. Plant Physiol. Mol. Biol.* **44**, 543 (1993).
8. C. Hornberg and E. W. Weiler, *Nature* **310**, 321 (1984).
9. M. Koornneef, G. Reuling, C. M. Karssen, *Physiol. Plant.* **61**, 377 (1984).
10. M. Koornneef and C. J. Hanhart, Arabidopsis *Inf. Serv.* **21**, 5 (1984).
11. Mapping was performed with molecular markers [C. Chang, J. L. Bowman, A. W. DeJohn, E. S. Lander, E. M. Meyerowitz, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6856 (1988); H.-G. Nam *et al.*, *Plant Cell* **1**, 699 (1989)] as mentioned by E. Grill and C. Somerville [in *Molecular Biology of Plant Development*, G. I. Jenkins and W. Schuch, Eds. (Company of Biologists, Cambridge, 1991)].
12. E. Grill and C. Somerville, *Mol. Gen. Genet.* **226**, 484 (1991); E. R. Ward and G. C. Jen, *Plant Mol. Biol.* **14**, 561 (1990).
13. Standard technical procedures were applied [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)].
14. S. Gibson and C. Somerville, in *Methods in* Arabidopsis *Research*, C. Koncz, N.-H. Chua, J. Schell, Eds. (World Scientific, Singapore, 1992), pp. 119–143.
15. K. Meyer and E. Grill, unpublished results.
16. Yeast chromosomes were isolated and separated as described (*12*) to purify YAC DNA from agarose slices by electroelution (*13*). After phenol extraction and partial Hind III restriction, the purified YAC DNA was cloned into the Hind III cloning site of the cosmid pBIC 20, and the DNA was packaged. The plasmid pBIC20 (*15*) is a 28-kb binary vector derived from the plasmid pLAFR3 [B. Staskawicz, D. Dahlbeck, N. Keen, C. Napoli, *J. Bacteriol.* **169**, 5789 (1987)]. After transfection of *E. coli* NM554 (Stratagene), 288 cosmid clones were analyzed by colony hybridization with purified YAC DNA as a probe, which was prehybridized [S. Baxendale, G. P. Bates, M. E. MacDonald, J. F. Gusella, H. Lehrach, *Nucleic Acids Res.* **19**, 6651 (1991)] in the presence of total wild-type yeast DNA (5 mg). Positive cosmids were selected, and the cloned fragments were assembled into a contig on the basis of their Hind III restriction map and cross-hybridization (*13*). Additional clones were identified from an *abi1* genomic library constructed in pBIC20 to fill in regions of poor overlap in the contig (*15*).
17. Cells of *A. tumefaciens* strain C58 pGV3850 [P. Zambryski *et al.*, *EMBO J.* **2**, 2143 (1983)] were electroporated according to the protocol of E. Grill and C. Somerville (unpublished results). Cells were grown to optical density at 600 nm (OD_{600}) = 1.5 in Luria Broth (Difco, Detroit, MI) medium (*13*) at 30°C and harvested by centrifugation (4000*g*, 10 min) at 4°C. The cells were washed three times in half the original volume by their gentle resuspension in ice-cold water and their subjection to pelleting as mentioned above. Finally, 0.4 ml of cell suspension adjusted to OD_{600} = 20 was mixed with 5 μl of DNA solution [0.1 μg of DNA in 10 mM tris-Cl, 1 mM ethylenediaminetetraacetic acid (pH 8.0)], electroporated (Bio-Rad electroporator; settings: 10 kV/cm, 1 kilohm, 25 μF), and immediately diluted with 1 ml of Luria Broth medium. After 3 hours of phenotypic expression at 30°C, the cells were transferred to selective media.
18. C. Simoens *et al.*, *Nucleic Acids Res.* **14**, 8073 (1986); H. J. Klee, M. B. Hayford, S. G. Rogers, *Mol. Gen. Genet.* **210**, 282 (1987).
19. Roots of *Arabidopsis*, ecotype RLD, were transformed with the use of *A. tumefaciens* strain C58 pGV 3850 containing pBIC20 cosmids. The transformation and regeneration of plants were carried out as described [D. Valvekens, M. Van Montagu, M. Van Lijsebettens, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536 (1988)] with the modifications that glucose was replaced by sucrose and that the concentration of kanamycin during plant regeneration was 25 mg per liter. Regenerates were either grown to maturity under sterile conditions or rooted and afterward cultivated in soil [H. Huang and H. Ma, *Plant Mol. Biol. Rep.* **10**, 372 (1992)].
20. An *Arabidopsis* (Landsberg *erecta*) cDNA library was constructed in lambda gt10 from 5 μg of poly(A)$^+$ RNA from 10-day-old sterile seedlings. The construction and screening of the library containing 3×10^6 primary plaque- forming units was performed according to standard procedures (*13*).
21. M. P. Leube, T. T. Ehrler, M. Iten, E. Grill, unpublished results.
22. Nucleotide sequences were determined on both strands with the use of the chain termination method and standard strategies (*13*). The genomic sequence was submitted to the European Molecular Biology Laboratory (EMBL), accession number X78886. The sequence of the 2.0-kb cDNA insert from clone λabi1-7 was determined and, in addition, the corresponding nucleotide sequence of the *abi1* mutant gene (including three introns of 70, 92, and 131 bp). The nucleotide sequence of the introns and the difference at the mutation site were verified by the sequencing of a genomic fragment (1.39 kb) of the wild-type *ABI1* allele, which was amplified by PCR [R. K. Saiki *et al.*, *Science* **230**, 1350 (1985)].
23. The sequence comparisons and searches in the databases of EMBL, GenBank, and Swissprot were performed with the program package that included FASTA, TFASTA, MOTIFS, BESTFIT, and PILEUP, 7.2; Genetics Computer Group Inc., Madison, WI.
24. M. P. Leube and E. Grill, unpublished results.
25. S. Tamura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1796 (1989).
26. T. Maeda, A. Y. M. Tsai, H. Saito, *Mol. Cell. Biol.* **13**, 5408 (1993).
27. J. M. Burns Jr., M. Parsons, D. E. Rosman, S. G. Reed, *J. Biol. Chem.* **268**, 17155 (1993).
28. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. P. Cohen, *Annu. Rev. Biochem.* **58**, 453 (1989).
30. S. J. O'Keefe, J. Tamura, R. L. Kincaid, M. J. Tocci, E. A. O'Neill, *Nature* **357**, 692 (1992); N. A. Clipstone and G. R. Crabtree, *ibid.*, p. 695.
31. S. Luan, W. Li, F. Rusnak, S. M. Assman, S. L. Schreiber, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2202 (1993).
32. F. Foor *et al.*, *Nature* **360**, 682 (1992).
33. M. A. Estelle and C. Somerville, *Mol. Gen. Genet.* **206**, 200 (1987).
34. S. Nakayama, N. D. Moncrief, R. H. Kretsinger, *J. Mol. Evol.* **34**, 416 (1992).
35. M. Saraste, P. R. Sibbald, A. Wittinghofer, *Trends Biochem. Sci.* **15**, 430 (1990).

36. We thank N. Armhein and C. Somerville for helpful discussion, M. Koornneef for the *abi1* mutant, R. Fries and S. Melzer for access to the DNA sequencing facility, and E. M. Meyerowitz and H. M. Goodman for RFLP markers. Supported by the Swiss National Science Foundation.

14 February 1994; accepted 26 April 1994

Estrogen Receptor–Associated Proteins: Possible Mediators of Hormone-Induced Transcription

Shlomit Halachmi, Emily Marden, Glover Martin, Heather MacKay, Ciro Abbondanza, Myles Brown*

The estrogen receptor is a transcription factor which, when bound to estradiol, binds DNA and regulates expression of estrogen-responsive genes. A 160-kilodalton estrogen receptor–associated protein, ERAP160, was identified that exhibits estradiol-dependent binding to the receptor. Mutational analysis of the receptor shows that its ability to activate transcription parallels its ability to bind ERAP160. Antiestrogens are unable to promote ERAP160 binding and can block the estrogen-dependent interaction of the receptor and ERAP160 in a dose-dependent manner. This evidence suggests that ERAP160 may mediate estradiol-dependent transcriptional activation by the estrogen receptor. Furthermore, the ability of antiestrogens to block estrogen receptor–ERAP160 complex formation could account for their therapeutic effects in breast cancer.

The estrogen receptor (ER) is a member of a superfamily of nuclear receptors for small hydrophobic ligands including the steroid hormones, thyroid hormone, vitamin D, and the retinoids (*1*). As a class, these receptors are transcription factors that are regulated allosterically by ligand binding. Extracellular estradiol freely diffuses across the cell membrane and binds ER, leading to ER dimerization and to tight binding of ER to its specific DNA target, the estrogen responsive element (ERE). After ERE binding, the liganded ER activates transcription by as yet unknown mechanisms. Although the targets of ER activation in breast cancer are unknown, they are believed to be critical for cellular proliferation because a large