ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in *Arabidopsis*

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Abstract The *abi2*-1 (abscisic acid insensitive) mutant of *Arabidopsis thaliana* shows abscisic acid (ABA) insensitivity with respect to seed germination and vegetative ABA responses. We identified the *ABI2* gene by a combination of positional mapping and homology to *ABI1*. The ABI2 protein shows 80% amino acid sequence identity to ABI1, a protein phosphatase 2C (PP2C) involved in ABA signaling. The mutation that confers the *abi2*-1 phenotype is equivalent to the mutation previously identified in *abi1*-1 and the resulting Gly^{168}Asp abi2 protein shows a reduced PP2C activity. Thus, a pair of highly homologous PP2Cs regulate ABA signaling.

Key words: Abscisic acid; Protein phosphatase 2C; Signal transduction; ABI2; ABI1

1. Introduction

The plant hormone abscisic acid (ABA) is involved in many aspects of plant growth and development, such as seed maturation and dormancy, as well as responses to environmental stresses such as cold, drought or salinity [1,2]. Upon water deficit, ABA levels increase [3,4] and the ABA signal induces stomatal closure thereby reducing transpirational water loss. Regulation of stomatal aperture is mediated by rapid and slow ionic currents in guard cells. The multiplicity of ionic currents triggered by ABA in guard cells has been profusely studied [5,6]. ABA also exerts an important effect through changes in gene expression [1]. Numerous genes are transcriptionally upregulated by ABA [7] and intense efforts have been made to characterize the *cis*- and *trans*-acting elements that mediate the ABA-induced changes at the transcriptional level [8–11]. In contrast, the elements of ABA perception and early signal transduction are yet unknown. As a common topic in signal transduction, protein phosphorylation also seems to play an important role in ABA signaling. Several protein kinases that are activated by ABA have been described [12–15]. On the other hand, phosphatases, as antagonists of kinases, are expected to serve an important function as well. Thus, ABI1, a protein phosphatase 2C (PP2C) with a distinctive N-terminal extension, was identified in the regulatory pathway of seed and vegetative ABA responses [16,17]. Second messengers such as pH, Ca^{2+} and inositol-1,4,5-trisphosphate have been implicated in ABA signaling [18,19]; however, the identification of additional components is required to unravel the molecular mechanism of the ABA action. In this scenario, *Arabidopsis* molecular genetics offers a powerful tool to discover new elements of the ABA signal transduction pathway. *Arabidopsis* mutants with reduced or enhanced sensitivity to ABA have been isolated and, in some cases, the affected gene has been identified. The loss-of-function mutation in the *era*1 locus, which encodes the β-subunit of a protein farnesyl transferase, confers an enhanced response to ABA in embryos [20]. Conversely, the *abi1*, *abi2* and *abi3* (abscisic acid insensitive) mutants show a reduced sensitivity to ABA [21]. ABI3, a transcriptional activator [22], acts in ABA signaling during seed development [23] while the *ABI1* and *ABI2* loci additionally mediate the ABA regulation of vegetative functions. The *abi1* and *abi2* mutants show common phenotypes in that both reveal a reduced dormancy of the seeds, a lesion in stomatal regulation, as well as a reduced inhibition of growth by ABA [21]. We report here the identification and characterization of the *ABI2* locus that was independently cloned recently [24]. The gene product of *ABI2* is 80% identical to ABI1 protein and the mutated abi2 protein reveals reduced protein phosphatase activity.

2. Materials and methods

2.1. Plant material

The *Arabidopsis thaliana* (L.) Heynh. ecotypes used in this study were the Landsberg *erecta* (La-er), Niederzenz (Ndz) and Columbia (Col) wild types. The *abi2* mutant derives from an ethylmethane sulfonate-mutagenized population of La-er M2 seeds and was kindly provided by Dr. M. Koornneef, Wageningen, The Netherlands. The *abi2* mutant was backcrossed to the wild type twice before experimental use to remove unlinked background mutations. *Arabidopsis* was grown at 22°C under a 16 h light, 8 h dark photoperiod at 100 μE m^{-2} s^{-1} and cultivated in a perlite-soil mixture.

2.2. Mapping techniques

In order to compare the segregation of the *abi2* mutation with respect to *Arabidopsis* molecular markers, 450 individual F_2 plants were generated from a cross between the homozygous *abi2* mutant (La-er ecotype) and a wild type (Ndz ecotype). Restriction fragment length polymorphism (RFLP) mapping was performed with molecular markers as described [25]. Screening of yeast artifical chromosome (YAC) libraries [26,27] was carried out according to [27].

2.3. Identification of cDNA and genomic clones

The cDNA and the genomic clones for *ABI2* were identified by standard techniques [28] using a 1.2 kbp *Sal*I-*Not*I fragment from the expressed sequence tag (EST) 166I6T7 (*Arabidopsis* Biological Resource Center, Columbus, OH) as a probe. The *ABI2* cDNA clone was isolated from an *Arabidopsis* (La-er) cDNA library constructed in λgt10 [17]. The 1.3 kbp *ABI2* cDNA was subcloned from λgt10 using PCR with *Pfu* DNA Polymerase (Stratagene, San Diego, CA) followed by blunt-end cloning into pBluescript SK (Stratagene) digested with *Eco*RV. The *ABI2* and *abi2* genomic clones were isolated from

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Arabidopsis (La-er) cosmid libraries constructed in the binary cosmid vector pBIC20 [29] from DNA of wild-type or *abi2* mutant plants, respectively. Screening of the library identified several overlapping cosmids. One pBIC20-*ABI2* clone from the wild-type library and one pBIC20-*abi2* clone from the *abi2* library with identical *Hin*dIII restriction patterns were selected for further analysis. These cosmids carried a genomic insert of approximately 17 kbp and the *ABI2* gene was contained within a 6.5 kbp *Hin*dIII fragment.

2.4. Introduction of the abi2 mutation into Columbia wild-type plants

The above described pBIC20-*ABI2* and pBIC20-*abi2* cosmids were independently introduced into *Agrobacterium tumefaciens* C58 pGV3850 [30] by electroporation [17]. Stability of the constructs was confirmed by *Hin*dIII restriction analysis of plasmid DNA isolated from transformed *A. tumefaciens*. *A. tumefaciens* containing the binary vector pBIC20-*ABI2* or pBIC20-*abi2* was used to transform Col wild-type plants by vacuum infiltration [31]. Transgenic lines were identified by selection for kanamycin resistance and β-glucuronidase activity [17].

2.5. Analysis of nucleic acids

Plant DNA, total yeast DNA and cosmid DNA were analyzed by Southern blot. Plant DNA was isolated from young leaves of *Arabidopsis* by the CTAB procedure [32]. Total DNA of yeast clones carrying YACs was prepared from 50 ml liquid cultures by the same procedure. DNA was digested with restriction endonucleases, transferred to Biodyne B membrane (Pall, Dreieich, Germany) and hybridized with the indicated probes according to Sambrook et al. [28]. Sequence analysis was performed on plasmid DNA or PCR products using T7 DNA polymerase and an ABI377 DNA sequencer. In order to obtain the *ABI2* and *abi2* genomic sequences the 6.5 kbp *Hin*dIII insert that hybridized to the *ABI2* cDNA was released from the pBIC20-*ABI2* or the pBIC20-*abi2* cosmid, respectively, and subcloned into pBluescript SK. Sequencing was performed with primers specific for the *ABI2* cDNA sequence and the data have been submitted to the EMBL database under accession number Y11840. A GGC to GAC transition at nucleotide 503 was found in the *abi2* genomic sequence. The mutation was also verified by the sequencing of a 1.6 kbp PCR-amplified genomic fragment of the *abi2*-1 allele. The mutation destroys a *Nco*I site (CCATGG→CCATGA), which was confirmed by Southern blot analysis of *abi2* DNA (La-er genetic background). Therefore, *Nco*I digestion of a 1.6 kbp *ABI2* genomic fragment amplified by PCR using Ndz DNA as a template produces two fragments of 500 and 1100 bp, whereas the *Nco*I site is absent when *abi2* DNA is used as a template. This difference provided the basis to develop a CAPS (cleaved amplified polymorphic sequences) marker [33] using the following PCR primers: 1, 5′-ATGGACGAAGTTTCTCCTGCAG-3′ and 2, 5′-CCTTCTTTTTCAATTCAAGG-3′.

2.6. Expression and purification of the ABI2 and Gly^{168}Asp abi2 proteins

The coding region of the wild-type *ABI2* cDNA was PCR amplified by using *Pfu* polymerase and primers 5′-AGCATGCAGGAAGTTTCTCCTGCAGTCGCT-3′ and 5′-GAGCTCGCATGCCATTCAAGGATTTGCTCTTGAA-3′. The amplified product was *Sph*I digested and cloned into the *Sph*I site of pQE70 (Qiagen, Hilden, Germany). The nucleotide change present in the *abi2*-1 mutation was introduced into the wild-type *ABI2* cDNA by site-directed mutagenesis [34] using the oligonucleotide 5′-TACGATGGCCATGACGGTTCTCAGG-3′. Once the constructs were verified by sequencing, they were used to transform the M15(pREP4) *Escherichia coli* strain (Qiagen). The expression of the recombinant proteins was induced with 1 mM IPTG and the ABI2 and the Gly^{168}Asp abi2 proteins were subsequently affinity purified on Ni-NTA resin as indicated by the suppliers.

3. Results

3.1. Positional mapping of the ABI2 locus

The *abi2*-1 mutation provides a semidominant ABA insensitive phenotype with respect to the inhibition of root growth by ABA. Root growth in the presence of 30 μM ABA was scored [17] in order to follow the segregation of the *ABI2* locus with respect to *Arabidopsis* molecular markers dispersed over the genome. Thus, the *ABI2* locus was localized to chromosome 5 by segregational analysis. The marker m558 cosegregated with the *ABI2* locus. Only five recombination events among 900 F_2 chromosomes analyzed were identified between the locus and the marker m558, reflecting a genetic distance of approximately 0.5 cM. The m558 marker is contained within a YAC contig generated by R. Schmidt et al. [35]. We verified the contig data by using the m558 marker for screening YAC libraries of *Arabidopsis* [26,27] and, as a result, the hybridization of CIC9E2, CIC9F3, CIC10B4, CIC9C9 and EG13G1 YACs to m558 was observed. The YAC contig extends about 560 kbp proximal (centromeric) and 400 kbp distal (telomeric) to the marker m558 (Fig. 1a). Taking into account the average value of 185 kbp per cM for the *Arabi-*

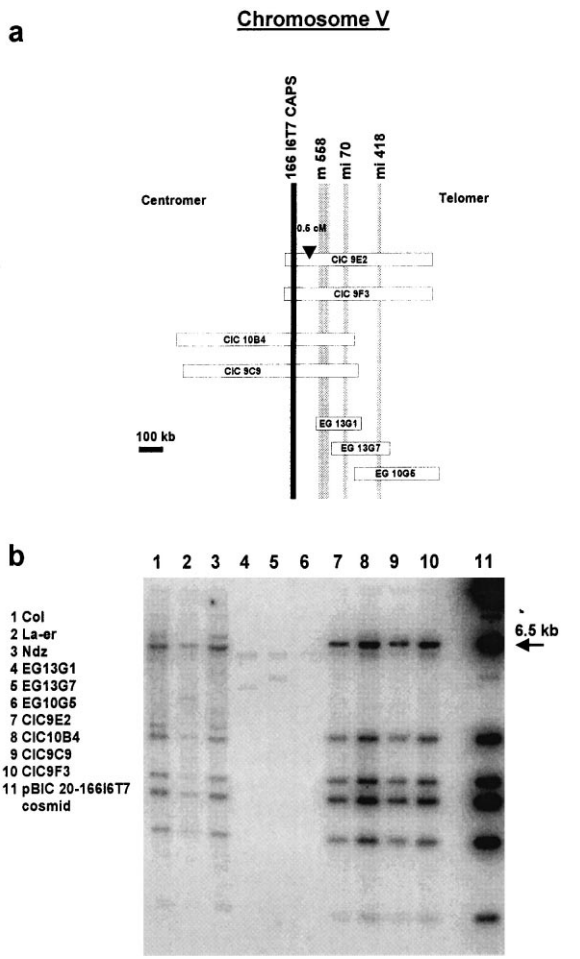

Fig. 1. a: Physical map of the *ABI2* locus relative to the YAC contig. Vertical bars represent polymorphic DNA fragments used to localize recombination breakpoints and to establish the YAC contig [35]. The 166I6T7 CAPS marker is described in the text and it represents the *ABI2* gene itself (see text). Inverted triangle indicates the closest breakpoint between the *ABI2* locus and m558. b: Autoradiogram of a Southern blot containing plant DNA, yeast DNA and cosmid DNA digested with *Hin*dIII and probed with radiolabeled pBIC20-166I6T7 cosmid DNA under high stringency conditions. Lanes 1–3 contain approximately 1 μg of Columbia, La-er and Niederzenz plant DNA, respectively; lanes 4–10 contain 0.3 μg of total DNA of yeast clones carrying the indicated YAC, respectively, and lane 11 contains 2 ng of pBIC20-166I6T7 DNA. The pBIC20-166I6T7 cosmid contains the *ABI2* gene in the 6.5 kbp *Hin*dIII fragment (see text) indicated by an arrow.

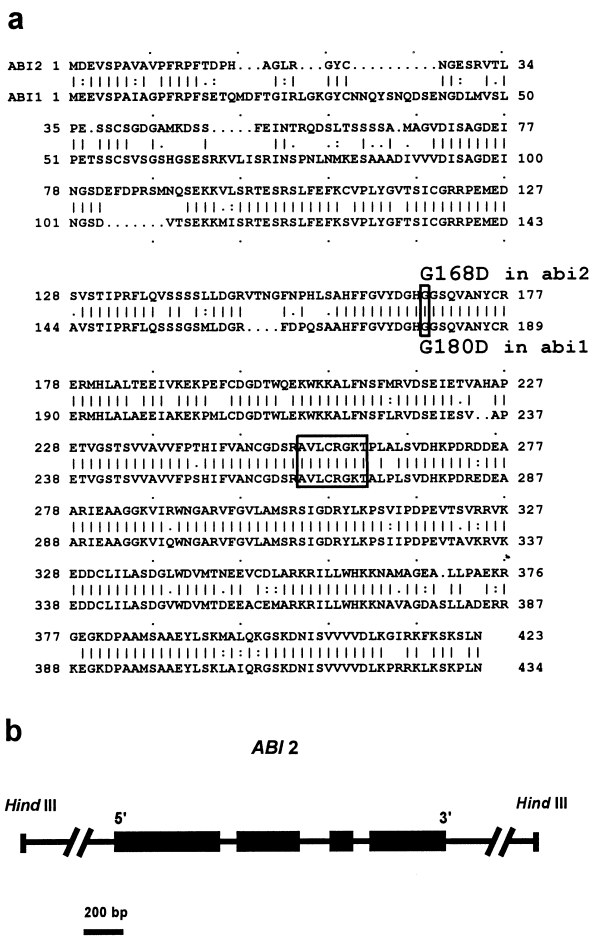

Fig. 2. a: Comparison between the predicted amino acid sequences of the ABI2 and the ABI1 proteins. The substitution of Gly by Asp in the abi2 and abi1 mutant proteins is emphasized. A putative ATP or GTP binding site [52], AVLCRGKT, is framed by a box. The sequence comparisons and searches in the databases were performed with the GCG package of the University of Wisconsin, Madison, WI, USA. b: Schematic diagram of the *ABI2* gene. Solid bars represent exons. Broken lines are introduced outside the *ABI2* gene to keep the scale. The *ABI2* gene was recovered as a 6.5 kbp *Hin*dIII fragment released from the cosmid pBIC20-166I6T7.
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dopsis chromosome 4 [36] as well as the 0.5 cM genetic distance of the *ABI2* locus with respect to m558, we reasoned that the locus should be contained within the YAC contig.

3.2. Cloning of an ABI1-like gene

The identification of ABI1 as a PP2C involved in ABA signal transduction raised the question of whether additional PP2Cs might also operate in ABA signaling. Several entries with homology to ABI1 are present in the *Arabidopsis* EST database and precedents for genetic complexity, i.e. partially redundant function of PP2C, are also found in yeast [37,38]. To investigate the above question, we decided to clone and map genes with homology to *ABI1*. To this end, *Arabidopsis* ESTs showing similarity to *ABI1* were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH). Among the ESTs that show similarity to *ABI1*, EST 166I6T7 presents the highest homology. The insert of clone 166I6T7 was used as a probe for screening a λgt10 cDNA library and a pBIC20 genomic library. A full length 166I6T7 cDNA encoding a protein with 80% amino acid sequence identity (84% similarity) to ABI1 was obtained (Fig. 2a). Sequence analysis of the 166I6T7 cDNA reveals a 1272 bp ORF that encodes a protein with 423 amino acids and a predicted molecular weight of 46 kDa. The homology to ABI1

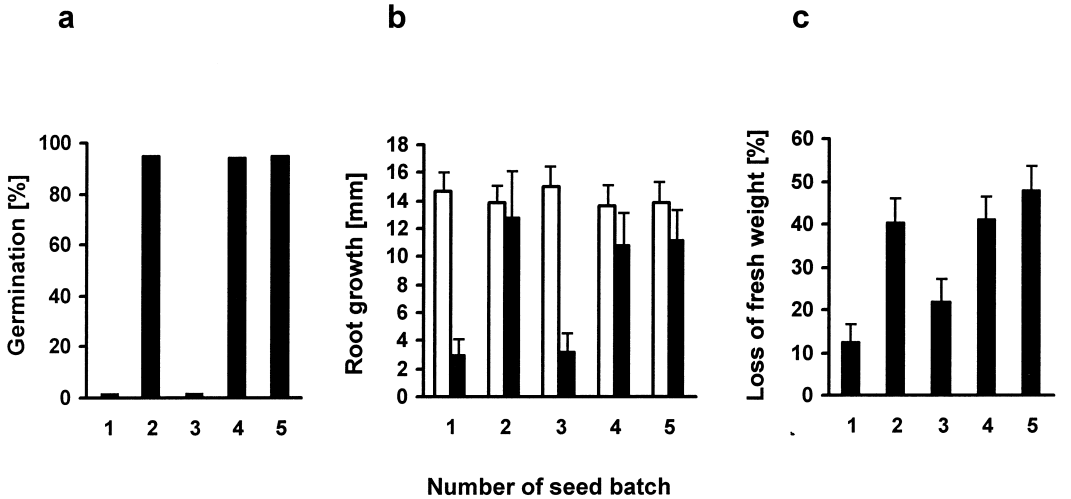

Fig. 3. Analysis of ABA-mediated responses. a: Seed germination in the presence of 3 μM (*R,S*)-ABA (Fluka) determined from 50 seeds of La-er (1), *abi2* mutant (2), Col (3), line 1 Col::T-DNApBIC20-*abi2* (4), line 2 Col::T-DNApBIC20-*abi2* (5) after 5 days. b: Root growth assay for scoring ABA sensitivity. The root growth was determined after 4 days of the transfer of 5-day-old seedlings of La-er (1), *abi2* mutant (2), Col (3), lane 1 Col::T-DNApBIC20-*abi2* (4) and lane 2 Col::T-DNApBIC20-*abi2* (5) onto MS plates containing 30 μM ABA (filled bars) or onto MS medium without ABA (open bars). The result represents the average growth including S.E. of 14 seedlings each analyzed as mentioned in [17]. c: Stomatal response analyzed by the desiccation of excised leaves at room temperature. Four leaves at approximately the same developmental stage and size from single 3-week-old plants of La-er (1), *abi2* mutant (2), Col (3), line 1 Col::T-DNApBIC20-*abi2* (4), line 2 Col::T-DNApBIC20-*abi2* (5) were excised and the loss of fresh weight was determined at ambient conditions after 20 min. The assay was performed with three single plants each.

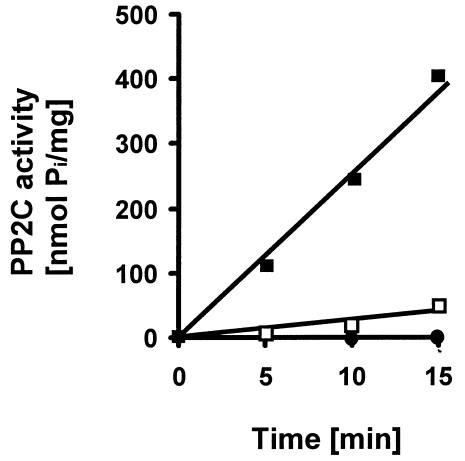

Fig. 4. PP2C activity of wild-type ABI2 protein and $Gly^{168}Asp$ abi2 protein. ABI2 (■) and abi2 (□) recombinant proteins (40 ng) were incubated for the indicated period of time at 30°C with ^{33}P-labeled casein in a buffer containing 30 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol and 20 mM magnesium acetate. In the absence of Mg^{2+} ions, both ABI2 and abi2 lack detectable phosphatase activity (●). The experiment was performed in duplicate and the phosphatase activity is expressed as nmol of inorganic phosphate released per mg of protein.

is not restricted to the PP2C domain. In fact, it extends to the distinctive N-terminal extension. The genomic sequence of the 166I6T7 gene was also determined. Comparison between the genomic and the cDNA sequences reveals a gene composed of four exons and three introns (Fig. 2b). The exon-intron junctions are located at positions equivalent to those found in the *ABI1* gene.

3.3. The ABI1-like gene represents ABI2

Screening of the genomic library provided a pBIC20 cosmid clone that contained the 166I6T7 gene in a 6.5 kbp *Hin*dIII fragment (Fig. 1b). In order to position the gene in the *Arabidopsis* genome, we used the cosmid clone to probe the *Arabidopsis* CIC library. The cosmid probe hybridizes to the CIC9E2, CIC9F3, CIC10B4 and CIC9C9 YACs (Fig. 1b) that constitute the previously confirmed YAC contig. These data, taken together with the high similarity of the gene to *ABI1*, prompted us to examine whether the 166I6T7 gene was indeed *ABI2*. A cosmid clone that hybridized to 166I6T7 was isolated from an *abi2* genomic library (see Section 2) constructed in the transformation-competent pBIC20 vector. The region of the cosmid clone that contains the 166I6T7 gene was sequenced. A single base pair difference was found with respect to the cDNA and the genomic wild-type clones. A GGC to GAC transition was identified leading to the replacement of the wild-type Gly^{168} residue by Asp (Fig. 2a). An identical mutation occurs in the *abi1*-1 mutant resulting in the substitution of the equivalent Gly^{180} residue by Asp (Fig. 2a). The mutation destroys a *Nco*I site, so a CAPS marker was developed using primers based on the sequence of the 166I6T7 gene. In this way, we could map the 166I6T7 gene with respect to the *abi2* locus. No recombination event was detected in the 900 F_2 chromosomes analyzed. The 166I6T7 gene cosegregated with the *abi2* locus. These data and the identification of a mutation support the conclusion that the 166I6T7 gene represents the *ABI2* gene. To further confirm the identity of the *ABI2* gene a pBIC20-*abi2* cosmid was introduced into Col wild-type plants by vacuum infiltration [31]. Two β-glucuronidase-positive transformants (T_0) were identified by kanamycin selection after screening approximately 40 000 seeds derived from infiltrated plants. Seeds of the individual transgenic lines (T_1 seeds) were tested for ABA insensitivity by examining their ability to germinate in the presence of 3 μM ABA, a concentration completely inhibitory to wild-type seeds in this assay (Fig. 3a). Seeds (T_1) of both transgenic lines containing the pBIC20-*abi2* T-DNA, however, germinated with frequencies of 94% and 96%, respectively, as well as the *abi2* mutant seeds (Fig. 3a). No segregation of the kanamycin resistance could be detected in the transgenic seedlings (T_1) indicating that the original transformed plants had integrated two or more unlinked copies of the T-DNA. In addition the T_1 progeny showed ABA insensitivity in both root growth (Fig. 3b) and stomatal response (Fig. 3c) comparable to the *abi2* mutant. Two independent lines transformed with the pBIC20-*ABI2* wild-type gene did not document an insensitivity to ABA (data not shown). Clearly, the introduction of the *abi2* gene into Col wild-type plants confers ABA insensitivity.

3.4. The mutant abi2 protein shows a reduced PP2C activity

To confirm that ABI2 does indeed show PP2C activity and to ascertain the effect of the *abi2*-1 mutation on the activity of the protein product, the wild-type ABI2 and the $Gly^{168}Asp$ abi2 proteins were expressed in *E. coli* as histidine tag fusion proteins. The heterologously expressed proteins were purified by Ni-NTA affinity chromatography and the phosphatase activity was assayed using ^{33}P-labeled casein as a substrate [39]. The activity of both the ABI2 and abi2 proteins revealed the strict Mg^{2+} dependence (Fig. 4) that forms a diagnostic feature of PP2C [39]. The capability to dephosphorylate casein was notably diminished, however, by the $Gly^{168}Asp$ substitution present in the abi2 protein yielding an approximately 10-fold lower specific activity of the mutant enzyme (39 pkat/mg) versus the wild-type protein (420 pkat/mg).

4. Discussion

ABI1 and ABI2 represent a pair of highly homologous PP2Cs involved in ABA signaling. Genetic duplication followed by functional diversification has emerged as a common theme in many regulatory genes [40].

The identification of an identical amino acid exchange in ABI1 and ABI2 as well as the pleiotropic ABA insensitivity mediated by the mutations indicate a functional redundance of both PP2Cs. The introduction of the complete *abi2* gene into *Arabidopsis* wild-type plants conferred ABA insensitivity in all three responses tested, namely germination, root growth and stomatal water loss, as has been previously observed with *abi1* transgenic plants [17]. This finding is in accordance with the co-dominant phenotype of heterozygous *abi2*-1 plants and, thus, differs from the phenotypes of the transgenic lines recently reported [24]. In this case, seed dormancy, ABA-independent germination and transpirational water loss were not consistently affected in the transgenic lines. The variation in the different ABA phenotypes observed could reflect incomplete gene transfer resulting in the expression of truncated versions of the abi2 protein. In our study, this problem was avoided by selecting transgenic lines that express functional

markers flanking the introduced genomic DNA as an indication of complete gene transfer [17].

The functional redundance of ABI1 and ABI2 would provide a basis to explain the failure to isolate recessive *abi1* or *abi2* alleles. Alternatively, other unidentified PP2Cs or additional phosphatases could partially rescue *abi1* or *abi2* recessive loss-of-function mutations.

This situation is found, for example, in the PP2Cs of *Saccharomyces cerevisiae* [41]. In this scenario, only codominant mutations such as those present in the *abi1*-1 and *abi2*-1 mutants could generate a phenotype.

Interestingly, the *abi1*-1 and *abi2*-1 mutations result in the substitution of an equivalent Asp residue for Gly. This substitution occurs at a position equivalent to Ala^{63} of human PP2Cα that is located in a domain involved in metal ion coordination [42]. Thus, the *abi1*-1 and *abi2*-1 mutations might disrupt the conformation of the Mg^{2+} coordinating site resulting in the reduced catalytic activity observed in the abi1 [43] and abi2 proteins [24] (Fig. 4). The diminished catalytic activity suggests that the *abi1*-1 and *abi2*-1 mutations act dominant negatively [44]. The dominant insensitivity to ABA might occur by functional disregulation of a complex of interacting proteins, e.g. due to the competition of the wild-type and the enzymatically less active mutant protein for a substrate.

In spite of the high similarity of the ABI1 and ABI2 proteins, the equivalent nature of the mutation as well as the common mutant phenotypes, a differential response of the *abi1*-1 and the *abi2*-1 mutants has been observed. A difference was found during drought rhizogenesis [45] and in the pattern of expression of certain ABA-inducible or stress-inducible genes [46–48]. In addition, the restoration of ABA sensitivity of guard cells by kinase inhibitors [49] was different in the *abi1*-1 and the *abi2*-1 mutants pointing to, at least, partially different roles for both protein phosphatases. Thus, ABI1 and ABI2 seem to have specific functions that could be mediated by the non-catalytic and less conserved amino-terminal domain of the proteins corresponding to 51% identity among the first 90 amino acid residues of abi2. In addition, both proteins might exert a redundant function in a subset of developmental or cellular processes as exemplified by the ETR1 [50] and ERS [51] in the ethylene response pathway. Clearly, experiments aimed at identifying the cellular targets of the ABI1 and ABI2 proteins will help to elucidate their function.

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References

[1] Chandler, P.M. and Robertson, M. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 113–141.
[2] Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.-C., Bouvier-Durand, M. and Vartanian, N. (1994) Plant Mol. Biol. 26, 1557–1577.
[3] Cornish, K. and Zeevaart, J.A.D. (1985) Plant Physiol. 79, 653–658.
[4] Davis, W.J. and Zhang, J. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 55–76.
[5] Blatt, M.R. and Thiel, G. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 543–567.
[6] Ward, J.M., Pei, Z.-M. and Schroeder, J.I. (1995) Plant Cell 7, 833–844.
[7] Skriver, K. and Mundy, J. (1990) Plant Cell 2, 503–512.
[8] Guiltinan, M.J., Marcotte Jr., W.R. and Quatrano, R.S. (1990) Science 250, 267–271.
[9] McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. (1991) Cell 66, 895–905.
[10] Nantel, A. and Quatrano, R.S. (1996) J. Biol. Chem. 271, 31296–31305.
[11] Skriver, K., Olsen, F.L., Rogers, J.C. and Mundy, J. (1991) Proc. Natl. Acad. Sci. USA 88, 7266–7270.
[12] Anderberg, R.J. and Walker-Simmons, M.K. (1992) Proc. Natl. Acad. Sci. USA 89, 10183–10187.
[13] Hwang, I. and Goodman, H. (1995) Plant J. 8, 37–43.
[14] Knetsch, M.L.W., Wang, M., Snaar-Jagalska, B.E. and Heimovaara-Dijkstra, S. (1996) Plant Cell 8, 1061–1067.
[15] Li, J. and Assmann, S.M. (1996) Plant Cell 8, 2359–2368.
[16] Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994) Science 264, 1448–1452.
[17] Meyer, K., Leube, M.P. and Grill, E. (1994) Science 264, 1452–1455.
[18] Blatt, M.R., Thiel, G. and Trentham, D.R. (1990) Nature 346, 766–769.
[19] Irving, H.R., Gehring, C.A. and Parish, R.W. (1992) Proc. Natl. Acad. Sci. USA 89, 1790–1794.
[20] Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996) Science 273, 1239–1241.
[21] Koornneef, M., Reuling, G. and Karssen, C.M. (1984) Physiol. Plant. 61, 377–383.
[22] Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Plant Cell 4, 1251–1261.
[23] Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M. and Giraudat, J. (1994) Plant Cell 6, 1567–1582.
[24] Leung, J., Merlot, S. and Giraudat, J. (1997) Plant Cell 9, 759–771.
[25] Grill, E. and Somerville, C. (1991) in: Molecular Biology of Plant Development (Jenkins, G.I. and Schuch, W., Eds.), Company of Biologists, Cambridge.
[26] Creusot, F., Fouilloux, E., Dron, M., Lafleuriel, J., Picard, G., Billaut, A., Le Paslier, D., Cohen, D., Chabouté, M.-E., Durr, A., Fleck, J., Gigot, C., Camilleri, C., Bellini, C., Caboche, M. and Bouchez, D. (1995) Plant J. 8, 763–770.
[27] Grill, E. and Somerville, C. (1991) Mol. Gen. Genet. 226, 484–490.
[28] Sambrook, J., Fritsch, F.E. and Maniatis, T. (1989) in: Molecular Cloning (Ford, N., Nolan, C. and Ferguson, M., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[29] Meyer, K., Benning, G. and Grill, E. (1996) in: Genome Mapping in Plants (Paterson, A.H., Ed.), pp. 137–154, Landes, Austin, TX.
[30] Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. and Schell, J. (1983) EMBO J. 2, 2143–2150.
[31] Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J. and Staskawicz, B.J. (1994) Science 265, 1856–1859.
[32] Rogers, S.O. and Bendich, A.J. (1994) in: Plant Molecular Biology Manual D1, pp. 1–8, Kluwer Academic, Dordrecht.
[33] Konieczny, A. and Ausubel, F.M. (1993) Plant J. 4, 403–410.
[34] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[35] Schmidt, R., Love, K., West, J., Lenehan, Z. and Dean, C. (1997) Plant J. 11, 563–572.
[36] Schmidt, R., West, J., Love, K., Lenehan, Z., Lister, C., Thompson, H., Bouchez, D. and Dean, C. (1995) Science 270, 480–483.
[37] Maeda, T., Wurgler-Murphy, S.M. and Saito, H. (1994) Nature 369, 242–245.
[38] Shiozaki, K. and Russell, P. (1995) EMBO J. 14, 492–502.
[39] McGowan, C.H. and Cohen, P. (1988) Methods Enzymol. 159, 416–426.
[40] Pickett, F.B. and Meeks-Wagner, D.R. (1995) Plant Cell 7, 1347–1356.
[41] Maeda, T., Tsai, A.Y.M. and Saito, H. (1993) Mol. Cell. Biol. 13, 5408–5417.

[42] Das, A.K., Helps, N.R., Cohen, P.T.W. and Barford, D. (1996) EMBO J. 15, 6798–6809.
[43] Bertauche, N., Leung, J. and Giraudat, J. (1996) Eur. J. Biochem. 241, 193–200.
[44] Herskowitz, I. (1987) Nature 329, 219–222.
[45] Vartanian, N., Marcotte, L. and Giraudat, J. (1994) Plant Physiol. 104, 761–767.
[46] Gilmour, S.J. and Thomashow, M.F. (1991) Plant Mol. Biol. 17, 1233–1240.
[47] Gosti, F., Bertauche, N., Vartanian, N. and Giraudat, J. (1995) Mol. Gen. Genet. 246, 10–18.
[48] Söderman, E., Mattsson, J. and Engström, P. (1996) Plant J. 10, 375–381.
[49] Pei, Z.-M., Kuchitsu, K., Ward, J.M., Schwarz, M. and Schroeder, J.I. (1997) Plant Cell 9, 409–423.
[50] Chang, C., Kwok, S.F., Bleecker, A.B. and Meyerowitz, E.M. (1993) Science 262, 539–544.
[51] Hua, J., Chang, C., Sun, Q. and Meyerowitz, E.M. (1995) Science 270, 1712–1714.
[52] Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) Trends Biochem. Sci. 15, 430–434.