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ABI1 of *Arabidopsis* is a protein serine/threonine phosphatase highly regulated by the proton and magnesium ion concentration

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Abstract The plant hormone abscisic acid (ABA) mediates various responses such as stomatal closure, maintenance of seed dormancy, and inhibition of plant growth. All three responses are regulated by the *ABI1* gene product. The ABI1 protein (ABI1p) has been characterized as a protein serine/threonine phosphatase of type 2C that is highly affected in its activity by changes in the proton and magnesium ion concentrations. In the ABA-insensitive mutant *abi1* of *Arabidopsis thaliana* a single amino acid exchange in the primary structure results in both a dominant insensitive phenotype and a strongly reduced protein phosphatase activity in vitro by possibly impairing metal ion coordination.

Key words: Protein phosphatase 2C; Signal transduction; Stoma; Dominant negative phenotype; *Arabidopsis thaliana*

1. Introduction

The plant hormone abscisic acid (ABA) is a key regulator in plant embryo development and in the adaptation responses towards low water potential [1,2]. Distinct pathways of ABA action such as stomatal closure, growth inhibition and seed dormancy seem in part to be controlled by common steps in signal transduction as supported by the pleiotropic effects of ABA response mutants of *Arabidopsis* such as the ABA-insensitive *abi1* and *abi2* [3] as well as *gca1* and *gca2* (unpublished results). These loci represent candidate genes for ABA signaling components or, alternatively, could encode gene products involved in antagonizing the individual ABA responses. The *ABI1* as well as the *ABI2* gene have been isolated and preliminarily characterized as two homologous protein serine/threonine phosphatases of type 2C (PP2C) [4–8]. The loci of both mutants confer a co-dominant and pleiotropic insensitivity towards the hormonal signal. Interestingly, the gene products of both mutant genes revealed a reduced enzymatic activity [6–8] indicating a dominant negative regulation by the mutants abi1p and abi2p.

PP2Cs represent a class of unique protein serine/threonine phosphatases (PP) both in being strictly dependent on Mg^{2+} and in revealing no sequence homology to the three other structurally related major classes, namely the PPs of type 1, 2A, and 2B [9]. However, the analysis of the crystal structure of PP2C documented a similar three-dimensional architecture of all classes of PPs [10]. PP2Cs of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were identified to counteract the activation of distinct MAP kinase pathways involved in osmoregulation and cell wall biosynthesis [11,12]. The molecular targets of PP2Cs in higher eukaryotes are just beginning to emerge [13,14]. In *Arabidopsis*, PP2Cs represent a gene family that comprises the ABIps and KAPP, a member interacting with a putative receptor kinase [15]. In addition, another PP2C was identified by antagonizing the action of a cAMP-dependent protein kinase when heterologously expressed in yeast [16]. The mode(s) of ABI1p and ABI2p action have not been defined. Electrophysiological analysis in abi1p expressing stomatal cells documented the rescue of the ABA response by the application of protein kinase inhibitors [17,18] and thus indicate that the dephosphorylated status of ABA signaling components is required for the response.

The mutant and the wild-type ABI1 proteins differ in the substitution of a single amino acid residue ($Gly^{180}Asp$) in the phosphatase domain [4,5]. An amino-terminal domain unique to this PP2C contains a putative EF hand structure that could function as a high affinity Ca^{2+} binding site. Cytosolic concentrations of Ca^{2+} and the proton abundance have been attributed a role as secondary messengers or regulators of the ABA response [19,20]. Thus, a modulation of the enzyme activity by these ions was of particular interest. In this report we carried out a detailed biochemical analysis of the abi1p/ABI1p in order to contribute to the elucidation of its mode of action.

2. Materials and methods

2.1. Chemicals

Okadaic acid, calyculin A and dephosphorylated casein were purchased from Sigma. [γ-^{33}P]ATP was purchased from Amersham. All other chemicals were from Fluka and of the highest purity available.

2.2. Expression of ABI1p

All molecular biology techniques were according to [21] unless otherwise stated. The expression of ABI1p was performed as a fusion protein with the glutathione *S*-transferase ($ABIp^{GST}$) adjacent to a factor Xa cleavage site. The ABI1 cDNA [5] was cloned as a *Mse*I-*Vsp*I fragment starting from the ATG start codon to the nucleotides located 72 bp downstream of the stop codon into the *Vsp*I site of pBR322 generating pBRABI. From this construct the complete ABI1 coding region can be released as a *Vsp*I fragment that was introduced into the *Stu*I site of the expression vector pGEX-G [22] yielding pGEXABI. To introduce the abi1 mutation a DNA fragment was amplified by the polymerase chain reaction with the primer A 5′-ACGGTTCTCAGGTAGCGAACTATTG and 5′-CCCAAACCCC-GTCACTCG using pBRABI as a template. The *Xba*I-digested PCR fragment was ligated into the vector pGEXABI, which was cut with *Nco*I, blunted by filling in with Klenow polymerase and digested with *Xba*I giving pGEXabi. The *Xba*I-*Bcl*I fragment from pGEXabi containing the mutation was exchanged with the corresponding fragment of pBRABI to yield pBRabi, which was subsequently used a source for the mutant cDNA.

The $ABI1p^{HIS}$ and $abi1p^{HIS}$ fusion proteins were expressed in *Escherichia coli* by cloning the wild-type and mutant structural genes of

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This work is dedicated to Prof. Dr. M.H. Zenk on the occasion of his 65th birthday.

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the PP into the vector pQE60 (Qiagen, Hilden, Germany). The vector was cut with *Nco*I, treated with mung bean nuclease and then cut with *Bam*HI. The corresponding cDNAs were provided by a *Vsp*I-*Sac*I DNA fragment from pBRABI and pBRabi, respectively, that was blunted at the *Vsp*I cut end with mung bean nuclease, and by a second cDNA fragment amplified by primer A and the primer 5′-AAGGATCCGCTCTTGAGTTTCC in a PCR reaction using pBRABI as a template. The amplified fragment was cut with *Sac*I and *Bam*HI and ligated into the vector yielding pQEABI and pQEabi, respectively. The cloning steps changed the last four carboxy-terminal amino acids of the proteins into the decameric peptide sequence $GSRSH_6$ containing six histidine residues. The correct nucleotide sequences of the exchanged DNA fragments from the various constructs were verified by sequencing [23].

For the expression of the fusion proteins, an overnight culture of *E. coli* M15 [pREP4] cells containing the expression constructs were diluted 1:20 with fresh medium. When the culture reached an $OD_{600} = 1$, the cells were induced with 20 μM isopropyl-thio-β-D-galactopyranoside for 2 h and then harvested by centrifugation. The GST fusion proteins were isolated by resuspension of the cells in 20 ml phosphate buffer, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4)/g cell mass and by subsequent sonication for 20×4 s (intensity 80%, sonicator W-380, Ultrasonics, LSL Secfroid, Aclens, Switzerland). Further purification was carried out according to [24]. GSH Sepharose was synthesized as described in [25]. The isolation of the histidine-tagged fusion proteins was performed as described in [26].

2.3. Characterization of ABI1p

2.3.1. Enzymatic assay. The protein phosphatase activity was measured as described [27] with the following modifications. The enzyme (approximately 20 ng) was preincubated for 10 min in buffers containing the appropriate salts prior to addition of the radiolabeled substrate phosphocasein (15 μg protein; 0.1–1 μCi/mg casein). Enzymatic activity (p_{kat}) was expressed as the liberation of inorganic phosphate (pmol) from casein per second. A 2-bis[2-hydroxyethyl]amino-2-[hydroxymethyl]-1,3-propanediol-Cl buffer and Tris[hydroxymethyl]-aminomethane-Cl buffer at pH 5.7–7 and pH 7–10, respectively, were employed in the measurements of the pH dependence. The reactions (30 μl) were stopped by the addition of 70 μl of a slurry of 10 g charcoal and 0.31 g dextran in 100 ml 0.1 M sodium phosphate buffer (pH 7). The mixture was vortexed for 1 min and then centrifuged for 5 min at 14 000×*g*. 70 μl of the supernatant containing the released phosphate was counted in a Beckmann LS 1801 scintillation counter.

2.3.2. Gel filtration. Gel filtration was performed using a Zorbax SE-250 column (Dionex, Sarasin AG, Basel, Switzerland) connected to a FPLC system (Pharmacia LKB Biotechnology). The purified protein was chromatographed on the column using the buffer 50 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM DTT at a flow rate of 0.5 ml/min. Fractions of 0.2 ml were collected and analyzed for protein phosphatase activity.

2.3.3. Equilibrium dialysis. Equilibrium dialysis was performed with 10 μg of the purified enzymes in one of two chambers separated by a semipermeable membrane of the Dianorm apparatus (Munich, Germany) [28]. The PP2C was equilibrated against the dialysis buffer containing 50 mM Tris-HCl (pH 8), 50 mM KCl, 5 mM $Mg(OAc)_2$ and 1 mg/ml bovine serum albumin prior to the experiment. The binding studies were conducted by the addition of either [^{32}P]α-ATP, [^{3}H]ABA or $^{45}CaCl_2$ to one of the two chambers. After 4 h of equilibration, aliquots were analyzed for the distribution of the radiolabeled compounds by scintillation counting (Beckmann LS 1801).

3. Results

3.1. Expression of mutant and wild-type enzyme

The wild-type ABI1p as well as the mutant abi1p were heterologously expressed in *E. coli* in order to characterize and compare their enzymatic properties in detail. The expression was performed both as a fusion protein of the glutathione *S*-transferase ($ABI1p^{GST}$ and $abi1p^{GST}$), and as proteins with a carboxy-terminal tag of hexameric histidine residues ($ABI1p^{HIS}$ and $abi1p^{HIS}$) to allow the affinity purification of the PP2Cs. Attempts to liberate the ABI1p from the GST fusion proteins via a specific endopeptidase cleavage site were unsatisfactory due to the generation of multiple cleavage products. Thus, the two expressed proteins differ from the native forms by either an amino-terminal or a carboxy-terminal modification, respectively.

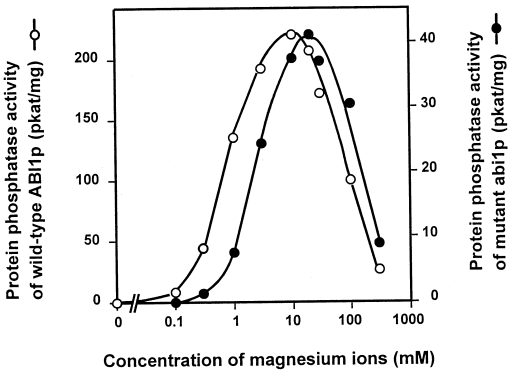

Fig. 1. Mg^{2+} dependence of protein phosphatase activity of wild-type $ABI1p^{GST}$ (○) and mutant $abi1p^{GST}$ (●) fusion proteins.

The proteins were expressed at relatively low levels affording approximately 1 mg of purified product per liter of bacterial culture. The enzymatic activities of the expressed proteins revealed the typical characteristics of PP2Cs, i.e. the dephosphorylation of the substrate phosphocasein was strictly dependent on the presence of Mg^{2+} (Fig. 1). The specific activities of the wild-type proteins $ABI1p^{GST}$ and $ABI1p^{HIS}$ were comparable with 220 and 240 p_{kat}/mg, respectively, in a standard assay system providing 20 mM Mg^{2+} at pH 8. The results indicate that the protein fusions do not negatively affect the performance of the phosphatase. The mutant forms $abi1p^{GST}$ and $abi1p^{HIS}$, however, yielded five-fold lower levels of enzymatic activity (41 and 50 p_{kat}/mg respectively). In accordance with previous studies on PP2C, Mg^{2+} can be substituted by Mn^{2+} [9]. $ABI1p^{GST}$ yielded 60% of enzymatic activity when 10 mM Mg^{2+} was replaced with 10 mM Mn^{2+}. No activity could be recovered by substituting with Ca^{2+}, Co^{2+}, Fe^{2+} or Zn^{2+} (10 mM). In addition, inhibitors of PP1 and PP2A, okadaic acid and calyculin A in concentrations of 10 μM and 1 μM, respectively, failed to block ABI1 activity.

3.2. Characterization of wild-type and mutant protein phosphatases

The amino acid substitution of Gly^{180} by an aspartic acid residue in abi1p is located within a highly conserved domain that is involved in complexation of Mg^{2+} [10]. Thus, the reduced activity of the abi1p observed could reflect impaired binding of the metal. The activation analysis of the metal-free enzyme by Mg^{2+} did indeed document a shift to higher Mg^{2+} concentrations required for the mutant protein compared to the wild-type form (Fig. 1).

The enzymatic activity of $ABI1p^{GST}$ was linearly enhanced up to an ion concentration of approximately 1 mM Mg^{2+} while the mutant protein required three times higher ion concentrations to achieve an equal level of activation. Half-maximal activation of the enzyme occurred at 0.8 and 2.4 mM Mg^{2+} for the wild-type and mutant form, respectively, with optimal values at 10–20 mM. Even at optimal Mg^{2+} concentrations, the enzymatic activity of the mutant protein was five-

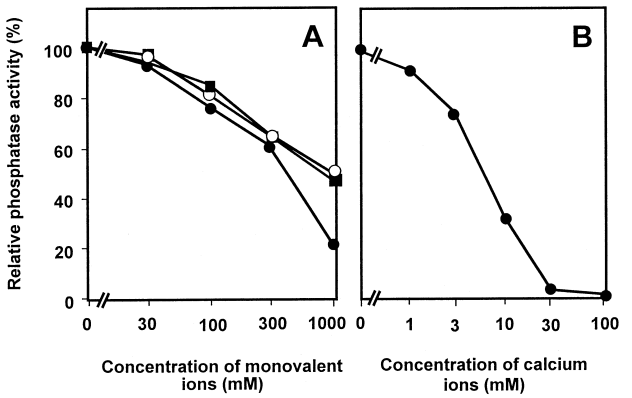

Fig. 2. Inhibition of ABI1 activity by the ionic strength in the presence of 10 mM magnesium acetate. A: Modulation of the phosphatase activity of ABI1p^{GST} fusion protein in the presence of the monovalent ions potassium acetate (●), sodium chloride (○) and potassium chloride (■). B: Inhibition of ABI1 activity by calcium acetate (●).

fold lower (Fig. 1). Parallel studies with ABI1p^{HIS} and abi1p^{HIS} yielded indistinguishable results.

However, concentrations of Mg^{2+} above the optimal levels resulted in a pronounced reduction in enzymatic activity. A 50% inhibition of ABI1p^{GST} (Fig. 1) and ABI1p^{HIS} was observed in the presence of approximately 80 mM Mg^{2+}. This inhibitory action does not seem to be ion-specific but rather to reflect a high sensitivity of the PP2C towards the ionic strength prevailing in the incubation medium. The presence of 80 mM magnesium acetate generates an ionic strength of approximately 240 mM. Substituting the magnesium acetate with monovalent potassium nitrate or sodium chloride (210 mM) in order to maintain the ionic strength under optimal Mg^{2+} ion concentration (10 mM) resulted in comparable enzymatic inactivation of the protein of 39% and 43%, respectively (Fig. 2A).

In addition, Ca^{2+} replacing the monovalent ions was even more inhibitory with a half-maximal rate at a concentration of 8 mM $CaCl_2$ corresponding to an ionic strength of 24 mM (Fig. 2B). Physiological Ca^{2+} levels in the range of 0.1–10 μM free Ca^{2+} did not affect the enzymatic activity, and attempts with the wild-type protein failed to assign a high affinity binding site for Ca^{2+} as implicated by the predicted EF hand structure. The analysis employed the highly sensitive equilibrium dialysis with $^{45}Ca^{2+}$ in the presence of ABI1p^{GST}. Likewise, binding studies with radiolabeled ATP in the same experimental system did not reveal any specific complexation to the PP2Cs and thus do not support the presence of a functional P-loop structure as a GTP/ATP binding site.

As cytosolic pH has been recognized as a central player in the regulation of potassium channels involved in stomatal regulation [19], the ABI1p activity as a function of pH was examined as well. Wild-type and mutant proteins had almost no activity at pH 6 while increasing pH from neutral to pH 8 resulted in a steep rise of enzyme activity as illustrated in Fig. 3. A further increase in pH resulted in a sharp drop of the enzymatic activity above pH 8. The mutant protein seemed to be even more sensitive to higher pH conditions. A shift in pH from 7.2 to 7.5 increased the specific activity of the enzyme by a factor of 2.2 in this in vitro experiment in the presence of optimal Mg^{2+} concentrations.

Under optimal assay conditions (pH 8.0; 20 mM Mg^{2+}), a

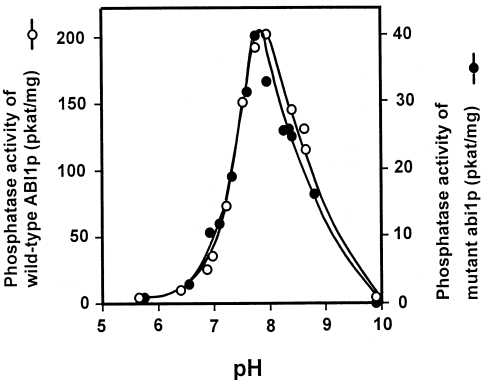

Fig. 3. Protein phosphatase activities of the wild-type ABI1p^{GST} (○) and the mutant abi1p^{GST} (●) fusion proteins as a function of pH. Assays were performed in the presence of 20 mM Mg^{2+}.

half-maximal rate of casein dephosphorylation was observed at 100 μM casein equaling 5.7 μM phosphoserine/threonine residues (Fig. 4). Due to the likely presence of some catalytically inactive PP2C in the preparation of ABI1p, a minimal dephosphorylation rate of 10^{-2} s^{-1} residue per enzyme molecule was determined under these conditions. The mutant protein revealed a five-fold reduced specific activity. Under physiological conditions with suboptimal Mg^{2+} concentrations, the activity would be further diminished by another factor of three to a residual activity of the approximately 6–7% of the wild-type enzyme.

These data and the dominant phenotype mediated by the abi1 locus support a dominant negative action of the gene product that could be generated by dysfunctional protein-abi1p complexes formed by either homomeric or heteromeric components. Gel filtration analysis was employed to examine the formation of homomeric PP2C complexes in vitro (Fig. 5). The analysis of ABI1p^{GST} and ABI1p^{HIS} clearly indicated in both experiments that the PP activity was only associated with the apparent molecular mass fractions of 67 kDa and 49 kDa, respectively, corresponding to the calculated molecular masses of monomeric proteins (70 and 48 kDa, respectively).

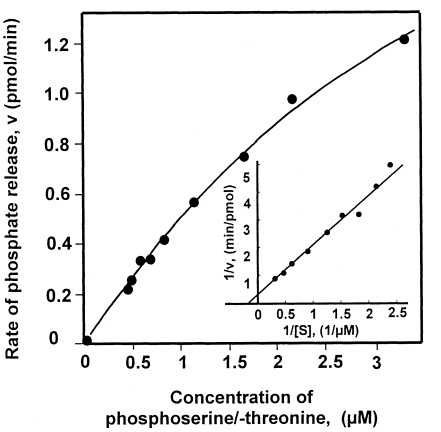

Fig. 4. Determination of the apparent K_m value of the PP2C. The insert depicts the reciprocal plot in the Lineweaver-Burk-diagram. An apparent K_m value of 5.7 ± 0.9 μM phosphoserine/threonine for the phosphorylated substrate casein was determined. Measurements were performed in the presence of ABI1p^{GST} and 20 mM Mg^{2+} in the reaction mixture.

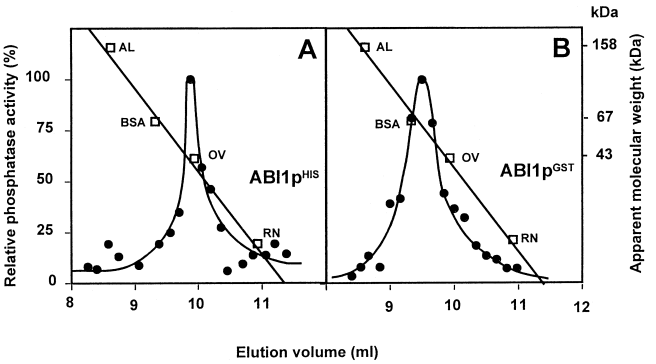

Fig. 5. Gel filtration analysis of the protein phosphatases ABI1p^{GST} and ABI1p^{HIS}. The apparent molecular weight of enzymatically active ABI1 protein fractions was determined after gel size exclusion chromatography. The column was calibrated with aldolase (AL, 158 kDa), bovine serum albumine (BSA, 67 kDa), ovalbumin (OV, 43 kDa) and ribonuclease A (RN, 14 kDa). The protein phosphatase activity (●) was maximal in a fraction corresponding to a M_r of 43 kDa for the ABI1p^{HIS} protein (calculated to equal 48 kDa) and of 62 kDa for the ABI1p^{GST} protein (calculated 75 kDa).

4. Discussion

The ABI1p controls several ABA-mediated responses involved in adaptations of the plant to a reduced water status such as stomatal closure. This process is characterized by initial membrane depolarization of guard cells within seconds after the ABA stimulus, followed by a phase of cytosolic alkalinization of up to 0.3 pH units and frequently by a concomitant increase of the free Ca^{2+} concentration in the cytosol [19,20]. This transient alkalinization of the cytosol would result in a pH-dependent activation of ABI1p. Thus, under conditions prevailing in the cytosol, i.e. pH 7.2 and 0.4 mM free Mg^{2+} [29], a shift to pH 7.5 would more than double the PP activity of ABI1p. Data on the ionic state of cytosolic Mg^{2+} in the initial phase and during the process of stomatal closing are not available, but a cytosolic increase of this ion by redistribution from the vacuole to the cytosol and finally to the apoplast during this response cannot be dismissed and could lead to further activation of ABI1p. Our data do not support the presence of a high affinity binding site for Ca^{2+} in the enzyme and a direct regulatory role of physiological concentrations of this ion is thus unlikely. Millimolar concentrations of Ca^{2+}, on the other hand, were found to deactivate the PP ([6], this study) possibly reflecting the competition for the Mg^{2+} binding site. The Asp^{60} residue is crucial for Mg^{2+} complexation in the human PP2C [10] corresponding to Asp^{177} in ABI1p [5]. Thus, the $Gly^{180}Asp$ exchange in the mutant protein is located in the vicinity of the Mg^{2+} coordination site and, indeed, affects the Mg^{2+}-dependent activation characteristics of the enzyme. The mutant protein required higher Mg^{2+} concentrations for activation and showed a 16-fold reduced phosphatase activity in the presence of physiological concentrations of this ion in vitro.

The strong modulation of ABI1p activity within a narrow pH range could provide a link to the role this enzyme serves in stomatal regulation. In addition, the finding that the mutant protein reveals a strongly reduced PP activity while mediating a dominant hormone insensitivity provides further support for the control of PP activity during the ABA response.

Two scenarios of ABI1p action can be visualized: the ABI1p could either be stimulated in the presence of ABA to act as a positive regulator of the hormone response or, alternatively, the enzyme exerts a negative control as postulated for the role of yeast PP2Cs on mitogen-activated kinase cascades [11,12]. The latter idea is in agreement with transient expression studies in maize protoplasts in which the ABA response was antagonized by enzymatically active ABI1p [30]. The dominant effect of the abi1p could be explained by a loss in the regulation of this signaling component, e.g. via interacting regulatory proteins or by an altered intracellular compartmentation. In both cases, a constitutive though lower activity of the mutant protein could be sufficient to continuously block the ABA response pathway.

A role of ABI1p as a positive effector could be envisaged on the basis of the ABA-mediated pH shift that results in the activation of ABI1p. The mutant protein would be less active and not efficiently transmit the signal. In accordance with this scenario, a rescue of the ABA response was observed in abi1p-expressing guard cells in the presence of a kinase inhibitor [17,18]. These studies also documented that the ABA-triggered alkalinization of the cytosol was not prevented by abi1p and, therefore, acts upstream of the PP in the signaling pathway. The dominant negative effect of the mutant enzyme could reflect the generation of a dysfunctional protein complex, e.g. by complex poisoning of a homomeric PP aggregate. PP2Cs are characterized, however, as monomeric enzymes [9] and our studies do not indicate the formation of homomeric Abi1p complexes. Thus, the dominant phenotype mediated by abi1p could reflect the involvement of a heteromeric protein association, e.g. a complex with a repressor of ABA signaling that is insufficiently deactivated by the reduced PP activity of abi1p. Interestingly, an inactive PP inhibited insulin signaling in a dominant negative manner probably by binding to and blocking the dephosphorylation of an interacting protein [31]. Similarly, the expression of a truncated PP resulted in a dominant negative hormone insensitivity by poisoning a receptor complex [32]. At this point, the identification of ABI1p interacting components and their characterization with respect to the interaction with the mutant and wild-type PP will be required to distinguish between the two models.

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