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A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in *Arabidopsis*

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Abstract Protein phosphatases of the 2C family (PP2C) function in the regulation of several signaling pathways from prokaryotes to eukaryotes. In *Arabidopsis thaliana*, the HAB1 PP2C is a negative regulator of the stress hormone abscisic acid (ABA) signaling. Here, we show that plants expressing a mutant form of HAB1 in which Gly246 was mutated to Asp (G246D) display strong ABA insensitive phenotypes. Our results indicate that the G246D mutation has a hypermorphic rather than a dominant negative effect. The data suggest that this mutation localized in a conserved motif in the PP2C catalytic domain could be used in other PP2Cs to reveal their biological functions.

Keywords: Protein phosphatase 2C; Abscisic acid; Dominant mutation; Hypermorphic

1. Introduction

Reversible protein phosphorylation regulated by protein kinases and protein phosphatases plays pivotal roles in the regulation of numerous biological processes. The understanding of the biological function of type 2C serine/threonine protein phosphatases (PP2C) has been further compromised because of the absence of specific inhibitors. Most of our knowledge on PP2C functions has been deduced from the characterization of mutants affecting PP2C genes in diverse organisms such as *rsbU* in *Bacillus subtilis* [1], *spalten* in *Dictyostelium* [2] or *fem2* in *Caenorhabditis elegans* [3]. However genetic analyses of PP2Cs function may be limited in some organisms that are not well suited for genetic manipulation or because of genetic redundancy.

In the reference plant *Arabidopsis thaliana* where 76 PP2C genes have been identified [4,5], the important role of 2 PP2Cs in the signaling pathway of the hormone abscisic acid (ABA) was first revealed by the isolation of two dominant mutants insensitive to this hormone. Interestingly, these two EMS-induced mutants carry equivalent mutations in 2 related PP2C genes called ABA insensitive 1 and 2 (*ABI1* and *ABI2*) [6–12]. These two mutations, *abi1-1* and *abi2-1*, respectively, correspond to the substitution of the same Gly for an Asp in a conserved motif of the PP2C catalytic site (Fig. 1). It was later shown that intragenic revertants of *abi1-1* mutants were slightly more sensitive to ABA than wild-type (WT). Because this phenotype was recessive (loss-of-function allele), these results indicated that ABI1 was a negative regulator of ABA signaling [11]. However, because the *abi1-1* mutation is still present in the intragenic revertant, this hypothesis was not unequivocally proven [11,13].

Here, we are interested in the *Arabidopsis* PP2C gene *HAB1* related to *ABI1* and *ABI2*, for which a knock-out allele, *hab1-1*, was isolated. This loss of function mutant is more sensitive to ABA compared to WT, undoubtedly demonstrating that HAB1 acts as a negative regulator of ABA signaling [14,15]. We introduced the *abi1-1*-like point mutation in the *HAB1* gene to analyze the exact effect of this mutation on the function of HAB1 *in planta*. We show that the Gly to Asp substitution in the catalytic site of this PP2C behaves as a dominant positive or hypermorphic mutation *in planta*. Using available data on PP2Cs we discuss different models that can account for this hypermorphic effect.

2. Materials and methods

2.1. Plant material, growth conditions and germination assay

Arabidopsis thaliana plants were grown in a growth chamber (22 °C, 75% RH, 16 h light photoperiod at 75 $\mu E\ m^{-2}\ s^{-1}$). High humidity condition culture was obtained by covering pots containing seeds with Saran wrap. For *in vitro* culture, sterilized-seeds from the same batches were plated on a solidified Murashige and Skoog medium (Sigma) supplemented with increasing ABA (Sigma, A1049) concentrations. After 4 days of stratification at 4 °C, seed germination was scored as fully expanded green cotyledons after 6 days of culture in the growth chamber.

2.2. Construction of transgenic plants

The pCambia1302-HAB1 construct made to express the *HAB1* cDNA under the control of the *Cauliflower mosaic virus* 35S promoter in plants was previously described in [14]. The substitution of Gly246 (codon GGA) to Asp (codon GAC) was introduced into the *HAB1* cDNA from pCambia1302-HAB1 using the mutagenic primers

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Abbreviations: ABA, abscisic acid; ABI, abscisic acid insensitive; LEA, late embryogenesis abundant; PP2C, protein phosphatase 2C; WT, wild-type

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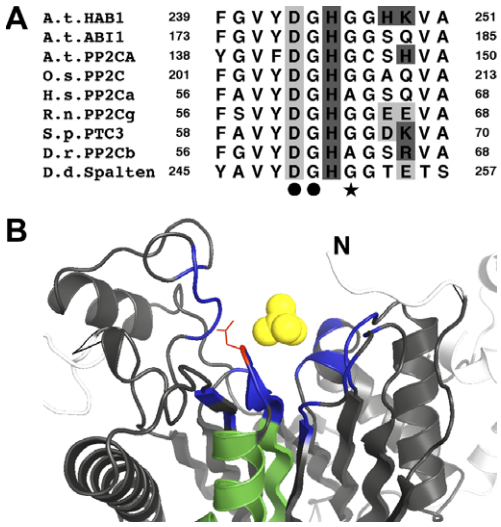

Fig. 1. (A) Sequences alignment around the conserved motif DGH of 9 PP2C from *Arabidopsis thaliana* (HAB1, GI:3242077; ABI1, GI:15236110; PP2CA, GI:15229745), *Oryza sativa* (GI:50901862), *Homo sapiens* (GI:38648670), *Rattus norvegicus* (GI:22219444), *Schizosaccharomyces pombe* (GI:1019405), *Danio rerio* (GI:51260856) and *Dictyostelium discoïdeum* (GI:2425121). Acidic and basic amino acids are identified by dark and light gray shading, respectively. Asterisk designates the position of the Gly residue converted to Asp in *hab1*^{G246D} and *abi1-1* mutants. Dark circle indicates the position of the metal coordinating residues. (B) 3D structure prediction of hab1^{G246D} catalytic domain. This model is based on a sequence alignment between hab1^{G246D} and the human PP2Cα obtained with the Phyre program (http://www.sbg.bio.ic.ac.uk/phyre/). The representation of the 3D structure was realized with the PyMOL program (http://pymol.sourceforge.net/). We obtained a good alignment in the PP2C catalytic domain between hab1^{G246D} and PP2Cα. More divergent regions are colored in white. The G246D mutation (red) is localized in a loop between sheet β4 and helix α1 (green). Residues localized 8 Å around the phosphate group (yellow spheres) are indicated in blue. The location of the N-terminal extension is indicated by the N letter.

5′-GTTTATGATGGTCATGACGGCCATAAGGTTGCT-3′ and 5′-AGCAACCTTATGGCCGTCATGACCATCATAAAC-3′. The resulting *hab1*^{G246D} cDNA was reamplified with primers 5′-ATCTCGAGATGGAGGAGATGACTCCCGCAGTT-3′ and 5′-ATGGATCCTCAGGTTCTGGTCTTGAACTTTCTT-3′ and cloned as a *Xho*I/*Bam*HI fragment in pKannibal vector [16]. The resulting *35S:hab1*^{G246D} cassette expressing *hab1*^{G246D} under the control of the 35S promoter was cloned as a *Not*I fragment into pArt27 binary vector [17]. Both pCambia1302-HAB1 and pArt27-*hab1*^{G246D} constructs were transformed in *Arabidopsis* Col-0 ecotype [18] and transgenic plants were selected on hygromycin (30 mg/L) and kanamycin (50 mg/L) containing medium, respectively. Seven independent *35S:hab1*^{G246D} primary transformants lines and three independent *35S:HAB1* lines were further analyzed. After self-fertilization, 70–100 T2 seeds were plated on medium supplemented with either 10 μM ABA or antibiotics. We selected the homozygous transgenic *hab1*^{G246D}#7 line for further analyses.

2.3. PP2C activity

HAB1 and *hab1*^{G246D} were PCR-amplified from pCambia1302-HAB1 [14] and pArt27-*hab1*^{G246D}, respectively, using the primers 5′-ATGGATCCATGGAGGAGATGACTCCCGCAGTT-3′ and 5′-ATCTCGAGGGTTCTGGTCTTGAACTTTCTTTGA-3′ and cloned as *Bam*HI/*Xho*I fragments in pGEX-KG vector [19]. The resulting GST fusion proteins were expressed in *Escherichia coli* strain BL21 Codon+ RIL (Stratagene) at 21 °C and purified on glutathione Sepharose 4B resin as indicated by the suppliers (Pharmacia). PP2C activity was determined using ^{32}P-labeled casein as an artificial substrate according to [11,20].

2.4. Stomatal aperture measurements and thermal imaging

Stomatal aperture measurements were performed on epidermal strips as described in [14]. Stomatal apertures (width and length) were measured so that the genotype or ABA concentration was unknown to the experimenter (double-blind experiments). Standard errors were calculated from three independent experiments with 120 stomata each analyzed. *t* Test (one-tailed, homoscedastic) *P*-values were calculated: $P = 2.0E-08$ for *hab1*^{G246D}#7 line at 0.1 μM ABA.

Thermal imaging of drought stress plants and analysis were performed exactly as described in [12].

2.5. Northern-blot and quantitative-PCR analyses

Total RNAs were extracted using Trizol reagent (Invitrogen). Northern blot analyses were realized using 15 μg of total RNA blotted onto a Hybond-N$^+$ membrane (Amersham-Pharmacia) and hybridized with ^{32}P-labeled DNA probes corresponding to *COR47* (At1g20440), *RAB18* (At5g66400), *HAB1* (At1g72770) and the putative late embryogenesis abundant (*LEA*) gene At1g52690.

Quantitative RT-PCR was performed on a Roche lightcycler system (Roche Diagnostics) according to the manufacturer's instructions, using 2.5 μg of total RNA and superscript reverse transcriptase II (Invitrogen). *ACTIN2* was used as an internal standard. The primer sets used to amplify *HAB1* were 5′-TGCGGTGATTCGAGGGCG-3′ and 5′-TTCCGGTTCTGGGATCACAT-3′ and for *ACTIN2* 5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′.

3. Results

3.1. Introducing the G246D mutation in HAB1

The dominant mutation *abi1-1* is a substitution of Gly180 (corresponding to G168 in ABI2) to Asp in the PP2C domain of ABI1 [7–10]. This Gly is in the midst of a very well conserved motif found in PP2Cs from *Arabidopsis* and other organisms (Fig. 1A). We introduced this mutation in the *HAB1* gene by site directed mutagenesis changing the codon GGA coding for Gly246 to GAC coding for Asp. In the following experiments this mutant form of *HAB1* will be referred as *hab1*^{G246D}.

3.2. The G246D mutation reduces HAB1 PP2C activity in vitro

We measured the PP2C activity of HAB1 *in vitro* using phospho-casein as substrate. As shown in Fig. 2, GST-HAB1 efficiently dephosphorylated casein with an apparent activity of 15.4 pmol Pi min^{-1} mg^{-1}. This phosphatase activity was strictly dependent on magnesium and insensitive to 10 μM okadaic acid (data not shown) which are the characteristics of PP2C activity [20]. In parallel experiments, we observed that

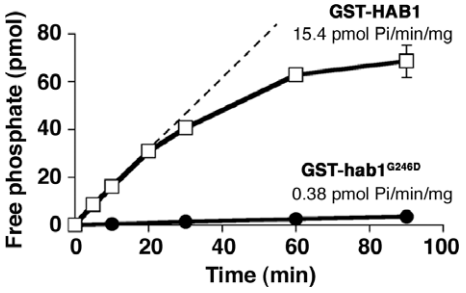

Fig. 2. Phosphatase activity of GST-hab1^{G246D} is reduced *in vitro*. GST-HAB1 and GST-hab1^{G246D} recombinant PP2C (100 ng) were incubated at 30 °C with ^{32}P-labeled casein for the indicated times in the presence of 20 mM magnesium acetate. Values shown are means ± S.D. of three replicate assays.