Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from *Arabidopsis*

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Abstract Protein phosphatases 2C (PP2Cs) exhibit diverse regulatory functions in signalling pathways of animals, yeast and plants. ABI1 is a PP2C of *Arabidopsis* that exerts negative control on signalling of the phytohormone abscisic acid (ABA). Characterisation of the redox sensitivity of ABI1 revealed a strong enzymatic inactivation by hydrogen peroxide (H_2O_2) which has recently been implicated as a secondary messenger of ABA signalling. H_2O_2 reversibly inhibited ABI1 activity in vitro with an IC_{50} of approximately 140 μM in the presence of physiological concentrations of glutathione. In addition, ABI1 was highly susceptible to inactivation by phenylarsine oxide (IC_{50} = 3–4 μM) indicative for the facile oxidation of vicinal cysteine residues. Thus, H_2O_2 generated during ABA signalling seems to inactivate the negative regulator of the ABA response.

Key words: Abscisic acid; Protein phosphatase 2C; Plant hormone; Signalling; *Arabidopsis*

1. Introduction

Protein phosphatases (PPs) constitute a diverse family of enzymes regulating a plethora of signalling pathways [1–3]. A variety of control mechanisms have been reported for the serine/threonine specific PP types PP1, PP2A and PP2B (calcineurin) [1,4,5] while little is known about the regulation of PP2Cs. PP2Cs are characterised as cytosolic enzymes with a strict requirement for Mg^{2+} [6]. Recently, unsaturated fatty acids emerged as regulators of PP2C from animal and plant [7,8].

Arabidopsis thaliana contains more than 70 genes with homology to PP2Cs [9]. Two of them, namely *ABI1* and *ABI2*, represent central regulators of signal transduction of the phytohormone abscisic acid (ABA) [10–14]. ABA is considered a stress hormone involved in regulation of plant's water status [15]. The phytohormone mediates closure of stomata and metabolic adjustments to water shortage. ABI1 and ABI2 share a high degree of structural identity in the catalytic domain (84%) and they contain a less conserved amino-terminal extension (42% identity in amino acid residues) of unknown function. The catalytic activity of ABI1 displays a strong dependence on pH and Mg^{2+} concentrations in the physiological range [16,17]. Transient expression analysis and intragenic suppressors of the ABA-insensitive *abi1-1* mutant indicate that ABI1 exerts a negative control on ABA signalling [18–20].

The recent characterisation of the cellular oxidant H_2O_2 as a second messenger in ABA signal transduction [21–23] prompted us to investigate whether ABI1 could be a target for the proposed redox signalling. Indeed, ABI1 displayed a high sensitivity to H_2O_2 probably by oxidation of adjacent cysteine residues. Thus, H_2O_2 could promote ABA signalling via inactivation of the negative regulator ABI1.

2. Materials and methods

2.1. Chemicals

Casein, protein kinase A and catalase coupled to agarose beads were obtained from Sigma, Germany. Methylumbelliferylphosphate (MUP), catalase from *Aspergillus niger* and all other chemicals of the highest purity available were purchased from Fluka, Germany.

2.2. Expression and purification of recombinant ABI1 protein

Expression of ABI1 carrying a carboxyl-terminal histidine tag (expression vector Qiagen pQE70) was performed as described previously [17]. The protein was purified by affinity chromatography on Ni^{2+}-NTA-agarose columns (Qiagen) according to the supplier's protocol. The ABI1 containing fractions were pooled and dialysed twice against 100-fold excess of 100 mM Tris–HCl, pH 8.0 (buffer A), supplemented with 10 mM β-mercaptoethanol and 20% glycerol. The PP2C was stored in aliquots at −80°C.

2.3. Incubations with H_2O_2 and phenylarsine oxide (PAO) and phosphatase assays

Purified ABI1 was concentrated by centrifugation in centrifugal filter devices (Ultrafree, Millipore; pore size 5K). Subsequently, aliquots were diluted 500-fold in buffer 50 mM Tris–HCl, pH 8.0, containing various concentrations of reduced glutathione (GSH). After the addition of H_2O_2 from calibrated stock solutions (ε = 44 M^{-1} cm^{-1} at 240 nm) or PAO the samples were incubated at 30°C for 60 min. The incubation was terminated by administration of catalase coupled to agarose beads (10 units; 5 min at 30°C). After the addition of Mg^{2+}-acetate to a final concentration of 20 mM the samples were preincubated for 5 min at 30°C and centrifuged (1 min, 12 000 × *g*). 20 μl of the supernatant was transferred to fresh tubes and the reaction was started by the addition of 10 μl solution containing phosphorylated casein at a final concentration equivalent to 2.4 μM phosphate. PP activity was assayed as described [17] except that bovine serum albumin was omitted from the assays.

Alternatively, a fluorimetric assay with MUP as substrate was used to determine ABI1 activity as described for difluoro-4-MUP [24].

Aliquots of ABI1 (0.8 μg in 15 μl) were mixed with Ni^{2+}-NTA-agarose (20 μl of a slurry in buffer A containing 20 mM β-mercaptoethanol) and gently agitated for 20 min at 30°C. The PP2C bound to the agarose beads was washed twice by dilution with degassed buffer

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Abbreviations: IC_{50}, inhibition constant; MUP, methylumbelliferylphosphate; PAO, phenylarsine oxide; PP, protein phosphatase; PP2C, protein phosphatase 2C

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A (1 ml) followed by centrifugation (12 000 × *g*; 20s) to remove β-mercaptoethanol and glycerol. After resuspension of the beads in 75 μl degassed buffer A containing 1 mM GSH the samples were incubated at 30°C in the presence of 0.3 mM H_2O_2 for the designated times. The inactivation was stopped by the addition of catalase (10 U). Finally, the agarose beads were resuspended to a homogenous slurry and 25 μl aliquots were transferred to microplates. Phosphatase activity was determined by recording the fluorescence of the product methylumbelliferone in a final volume of 100 μl containing 100 mM Tris–HCl, 1 mM MUP and 10 mM $MnCl_2$ in a microplate reader (Perkin-Elmer HTS 7000 Plus). Excitation and emission wavelengths were set at 360 nm and 465 nm, respectively.

3. Results

In the light of recent findings that H_2O_2 serves as a second messenger in ABA signal transduction [21–23] we investigated whether ABI1 could be regulated by H_2O_2. Indicative for a H_2O_2-mediated inactivation of ABI1 was the observation that purified ABI1 easily lost activity during storage which was recovered by incubation with the reductant dithiothreitol (data not shown). The reversible inactivation of the PP2C pointed to the oxidation of critical cysteine or methionine residue(s) [25]. In order to examine the sensitivity of ABI1 to H_2O_2, purified and freshly reduced ABI1 was incubated with increasing concentrations of H_2O_2 in the presence of 1 mM reduced GSH to mimic the cellular redox status [26,27]. The ubiquitous monothiol GSH represents the major cytosolic reductant and is known to be a scavenger of H_2O_2 [28]. Thus, the presence of GSH during oxidative ABI1 inactivation is detrimental for deducing a physiological significance. In fact, the PP2C was highly susceptible to H_2O_2 (Fig. 1). The enzymatic activity of ABI1 was reduced already at 100 μM H_2O_2 and dropped to half-maximal rates at approximately 140 μM oxidant. Concentrations of 0.3 and 1 mM H_2O_2 inhibited the enzyme more than 75% and 90%, respectively.

Analysis of the kinetics of the process revealed a loss of ABI1 activity within minutes after exposure to 0.3 mM H_2O_2 (Fig. 2A). ABI1 lost more than 65% of its enzymic activity within the first 10 min. Prolonged exposure resulted in the recovery of less than 20% of initial phosphatase activity after 30 min.

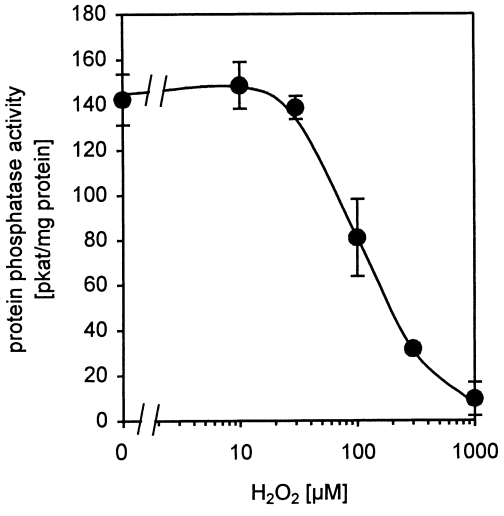

Fig. 1. Inactivation of ABI1 by hydrogen peroxide. ABI1 was incubated in the presence of various concentrations of H_2O_2 and 1 mM GSH and, subsequently, the phosphatase activity was determined. Data represent mean values ± S.D.

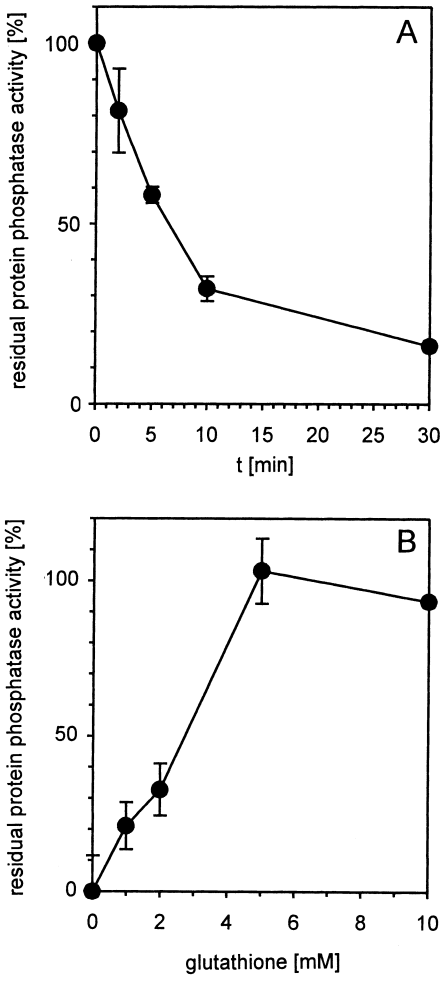

Fig. 2. Time course of ABI1 inactivation and dependence on glutathione levels. A: ABI1 is half-maximally inactivated within approximately 6 min in the presence of 300 μM H_2O_2. ABI1 was incubated for the designated times in the presence of 1 mM GSH prior to determination of residual phosphatase activity using the MUP assay. The initial activity ($t = 0$ min) was 517 pkat/mg protein. Data represent mean values ± S.D. B: The level of ABI1 inactivation by H_2O_2 is strongly influenced by GSH. After incubation of the enzyme with 300 μM H_2O_2 in the presence of different GSH levels for 1 h the residual ABI1 activity was determined. Data represent mean values ± S.D.

As mentioned above, the level and redox state of GSH probably influences the oxidative inactivation of ABI1. Thus, we examined whether GSH is an important factor in this process.

In the absence of GSH no ABI1 activity was recovered in the presence of 0.3 mM H_2O_2 after 1 h of incubation (Fig. 2B). In the presence of H_2O_2 and increasing GSH concentrations, however, the inhibitory effect was prominent only at low millimolar levels of GSH with less than 40% residual activity at 2 mM GSH. The presence of high GSH levels in the range of 5–10 mM prevented ABI1 inactivation.

The findings are compatible with a specific oxidation of cysteine or methionine residue(s). In a first experiment to investigate the possibility of intramolecular disulfide formation as the mode of inactivation we used the inhibitor PAO that specifically interferes with vicinal cysteine residues by forma-

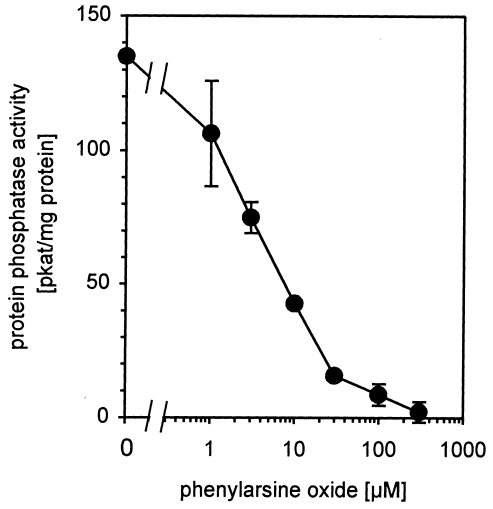

Fig. 3. Inactivation of ABI1 by PAO. ABI1 reveals an IC_{50} of 3 μM for the inhibitor PAO. For analysis, ABI1 was incubated in the presence of various concentrations of PAO and subsequently the PP activity was determined. Data represent mean values ± S.D.

tion of a cyclic adduct [29]. Treatment of ABI1 with increasing concentrations of PAO resulted in the efficient inactivation of ABI1 with an IC_{50} value of approximately 3 μM (Fig. 3). The inhibitory effect of PAO was reversible by incubation with dithiothreitol identical to the inactivation of ABI1 by H_2O_2 (data not shown).

4. Discussion

H_2O_2 and other reactive oxygen species have been identified as regulators of PPs such as protein phosphotyrosine phosphatases (PTPs) and calcineurin in the last years [29–40]. Among PP2Cs, ABI1 and its homologue ABI2 (unpublished results) seem to be the first members of this enzyme class that are sensitive to H_2O_2.

The susceptibility of PP to H_2O_2 provides a link between protein phosphorylation and the redox status of the cell [38]. In plants, perturbation of the cellular redox status is associated with oxidative bursts as a reaction to pathogen interaction [41–43] as well as with other stress responses such as photo oxidation [44] and wounding [45]. Recently, a role of H_2O_2 has emerged in responses mediated by the stress hormone ABA including stomatal closure [21–23]. ABA seems to trigger H_2O_2 accumulation which in turn is able to activate Ca^{2+} channels involved in stomatal closure [21].

The levels of H_2O_2 and GSH reported for plant tissues are compatible with an enzymatic inactivation of ABI1 by H_2O_2. The H_2O_2 concentrations reported vary and range up to 7 μmol/g fresh weight in *Arabidopsis* corresponding to a maximum of millimolar concentration without taking into account intracellular and extracellular compartmentation [44,46,47]. A concentration of 50 μM H_2O_2 was sufficient to initiate specific differentiation of plant cells [48] and to activate Ca^{2+} channels [21]. ABI1 was half-maximally inactivated by 140 μM H_2O_2 in the presence of GSH. A comparable degree of inhibition of PTPs [35] and of calcineurin [29,39] required 25 μM and 200 μM H_2O_2, respectively. Noteworthy, bovine PP2Cα, and other serine/threonine PPs were not effected by as much as 1 mM H_2O_2 [33]. The comparison underestimates the H_2O_2 sensitivity of ABI1 due to the protective GSH present in our analysis. The H_2O_2-mediated inactivation of ABI1 was evident up to 2 mM GSH (Fig. 2B). The cellular concentration of GSH is below or in the low millimolar range [27,28].

Inhibition of PPs by H_2O_2 has been presumed to reflect oxidation of two closely spaced cysteine residues [29,33,35]. In line with this model, the phenylarsonous acid derivative PAO that reacts with vicinal thiols is an efficient inhibitor of PTPs [30], calcineurin [29] and ABI1. Thus, the redox-sensitive inactivation of ABI1 probably reflects the formation of an intramolecular disulfide bridge interfering with catalysis. It is to early to speculate on putative target cysteine residues of ABI1 since there is only a single tertiary structure for a PP2C [49] available and the conservation of primary structures within the protein family is low.

The inactivation of ABI1 under oxidising conditions gains physiological importance in the light of ABA-mediated generation of H_2O_2 and a role of the PP2C as key regulator of ABA responses [10,11]. ABI1 has been characterised as a negative regulator of ABA signalling that requires the PP activity for function [18–20]. The generation of a transient oxidising environment by H_2O_2 during early ABA signalling [22,23] probably inactivates ABI1. The inactivation of the negative regulator would permit or enhance ABA responses. Subsequent cellular reduction of inactivated ABI1 and ABA-mediated induction of ABI1 gene expression [12,20] would restore the negative action of the PP2C. Thus, a regulatory loop is conceivable that is capable to modulate ABA signal transduction.

The immediate targets of ABI1 are still not known, however, PP2Cs antagonise mitogen-activated protein kinase (MAPK) pathways [50–52]. In plants, MAPK activation by H_2O_2 [53,54] and by ABA [55] could reflect the inactivation of ABI1 or of other redox-regulated PP2Cs [56]. Thus, the proposed mode of ABI1 regulation possibly represents a sophisticated means to integrate ABA signalling into the network of cellular redox homeostasis.

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