Redox Control of Protein Tyrosine Phosphatases and Mitogen-Activated Protein Kinases in Plants

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Environmental conditions, including light, temperature, water status, and soil salinity, all modify the redox state of plant cells (Allen et al., 1995). A number of studies have shown that oxidative stress is a common factor that affects plant growth and development under extreme environmental conditions (for review, see Mittler, 2002). Most recently, oxidative stress agent H_2O_2 has been shown to serve as a critical messenger molecule in many signal transduction pathways, including plant responses to pathogen, plant hormone abscisic acid, and abiotic stress factors (Wojtaszek, 1997; Pei et al., 2000; Bolwell et al., 2001; Mittler, 2002). At least one of the mechanisms underlying H_2O_2 function is the activation of calcium channels (Pei et al., 2000; Chico et al., 2002). Here, we report that plant Tyr phosphatases such as AtPTP1 (Xu et al., 1998) serve as targets for H_2O_2 and this may be associated with the regulation of mitogen-activated protein (MAP) kinase activity in plants.

Although Ser/Thr and Tyr kinases/phosphatases play a critical role in cell growth and development in animals, none of the typical Tyr kinases has been characterized from higher plants. The existence of Tyr phosphatases in plants has also been controversial until recently when several members of protein Tyr phosphatase (PTP) family were characterized from Arabidopsis (Gupta et al., 1998; Xu et al., 1998; Fordham-Skelton et al., 1999, 2002; Ulm et al., 2001, 2002; Gupta et al., 2002). These studies suggest that protein Tyr (de) phosphorylation performs critical functions in plants. In support of this hypothesis, biochemical and genetic analyses confirm that Tyr phosphorylation, like that in animals and fungi, is involved in the regulation of MAP kinase activities in plant cells (for review, see Tena et al., 2001; Zhang and Klessig, 2001; Luan, 2002; Ulm et al., 2002).

PTPs from all organisms contain an active site signature motif, (I/V) H**C**XAGXXR(S/T) G, which harbors the catalytic cysteinyl residue involved in formation of a phosphoenzyme reaction intermediate (Guan, 1994). The PTPs are categorized into three groups based on the studies in animals: receptor-like PTP, intracellular PTP, and dual-specificity PTP (DsPTP; Stone and Dixon, 1994). The receptor-like PTPs appear to be found in animals, but not in fungi and plants. The other two categories exist in all eukaryotes, including plants (Gupta et al., 1998, 2002; Xu et al., 1998; Fordham-Skelton et al., 1999, 2002; Ulm et al., 2001, 2002). Cys residue in the catalytic domain is essential for the catalytic activity of PTPs from plants as well as animals (Gupta et al., 1998, 2002; Xu et al., 1998) In animals, it has been shown that oxidative stress inactivates the PTPs (Caselli et al., 1998; Denu and Tanner, 1998; Lee and Esselman, 2002). In one of the receptor-like PTPs, H_2O_2 induces a rapid and reversible catalytic Cys-dependent conformation change in vivo (Blanchetot et al., 2002). These studies indicate that the catalytic Cys must be in the reduced form for a PTP to be active. Because redox regulation is critically important in plant cell regulation, we hypothesized that plant PTPs may also be regulated by redox state of the Cys and thereby serving as a molecular target for oxidative stress.

To study the effect of H_2O_2 on plant PTPs, we analyzed the phosphatase activity of the purified AtPTP1 using pyronitrophenyl phosphate as a substrate (Xu et al., 1998). Results from one representative experiment are shown in Figure 1. The phosphatase activity of AtPTP1 was linear with time until H_2O_2 (1 mM) was added to the assay mixture. The activity of AtPTP1 was arrested within 1 min after addition of H_2O_2 (Fig. 1A). The AtPTP1 remains inactive until 200 units of catalase and DTT (10 mM) was added to decompose H_2O_2 and reduce Cys residues in the AtPTP1 protein. The activity of AtPTP1 was rapidly restored within 4 min (Fig. 1A). To examine whether H_2O_2 affects the AtPTP1 stability, we treated purified AtPTP1 with H_2O_2 and/or DTT and/or catalase for 60 min, and samples were analyzed by SDS-PAGE. The data indicated that none of these treatments changed the AtPTP1 stability (Fig. 1B), suggesting that inactivation of AtPTP1 by H_2O_2 may follow the conformational change shown in animal PTPs (Blanchetot et al., 2002). Based on these experiments, we concluded that AtPTP1 and possibly other plant PTPs (containing the catalytic cysteines) are reversibly inactivated by oxidative stress, as shown in animal PTPs (Caselli et al., 1998; Denu and Tanner, 1998; Lee and Esselman, 2002).

In all organisms studied thus far, PTPs play a role in MAP kinase regulation. The activation of MAP kinases requires phosphorylation of Thr and Tyr res-

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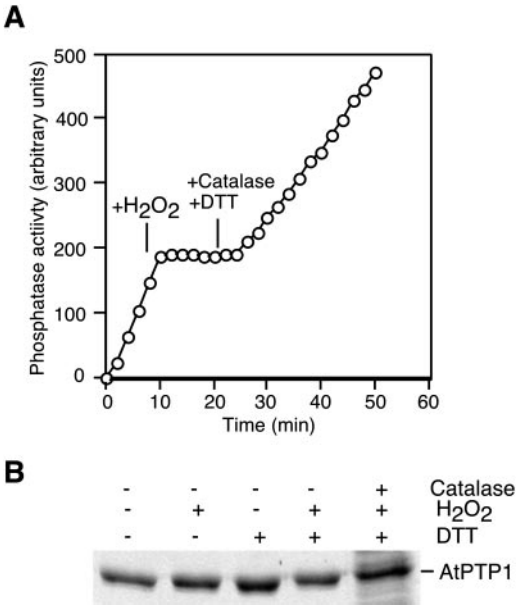

Figure 1. Effect of redox conditions on AtPTP1 protein and its phosphatase activity. A, Phosphatase activity of AtPTP1 (100 ng) was assayed at room temperature using pyronitrophenyl phosphate (20 mM) as a substrate in a phosphatase buffer (50 mM Tris-HCl, pH 7.0, and 2 mM dithiothreitol [DTT]) by measuring A_{405} as described previously (Xu et al., 1998). To change the redox state, H_2O_2 (1 mM) and DTT (10 mM and 200 units of catalase to decompose H_2O_2) were added 10 and 20 min after initiation of the phosphatase reaction. The data from one of three experiments are shown. Phosphatase activity calculated at different times is shown in arbitrary units. B, AtPTP1 is stable under different redox states. Purified AtPTP1 (5 μg) was incubated in 1 mM H_2O_2 and/or 10 mM DTT and/or catalase (200 units) for 60 min and was separated in a 12% (w/v) SDS-PAGE gel. The gel was stained with Coomassie Blue to detect AtPTP1.

idues in an activation loop of MAP kinases (Anderson et al., 1990). Dephosphorylation of Thr or Tyr or both causes inactivation of a MAP kinase (for review, see Camps et al., 2000; Keyse, 2000). In animals, dual-specificity PTPs (DsPTPs) have been shown to play a major role in the inactivation of different MAP kinases (for review, see Camps et al., 2000; Keyse, 2000). However, in yeast, DsPTP and Tyr-specific PTPs are involved in regulation of MAP kinases (Wurgler-Murphy et al., 1997; van Drogen and Peter, 2001). Recent studies have shown that PTPs function in the regulation of MAP kinases in plants as well (Luan et al., 2001; Luan, 2002; Ulm et al., 2002). Interestingly, oxidative stress such as H_2O_2 treatment activates MAP kinases in plant cells, consistent with the finding that H_2O_2 inactivates PTPs, negative regulators of MAP kinases. Although it is difficult to determine whether MAP kinase activation is a result of PTP inactivation by H_2O_2, we decided to make a correlation analysis.

To examine the MAP kinase activation pattern in Arabidopsis, we treated 3-week-old wild-type (Col-gl11) seedlings with H_2O_2 and measured MAP kinase activity in the total protein extract in an in-gel kinase assay (Zhang and Klessig, 1997). The H_2O_2 treatment activated a major MAP kinase at 47 kD within 5 min of treatment (Fig. 2A). Although earlier studies have

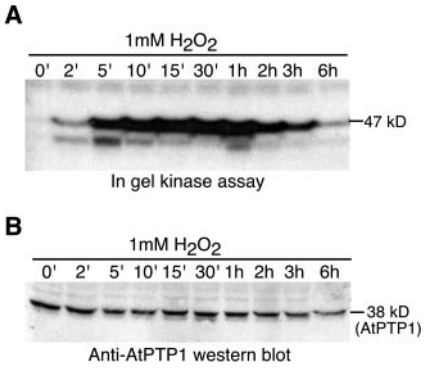

Figure 2. Oxidative stress activates a 47-kD (AtMPK6) MAP kinase without any change in AtPTP1 protein level. Three-week-old Arabidopsis (Col-gl11) seedlings grown on plates containing Murashige and Skoog medium (one-half strength Murashige and Skoog salt and 1× Gamborg's vitamins) were treated with 1 mM H_2O_2 (30 mL for each 90-mm plate) by submerging seedlings for different periods of time. Samples were frozen in liquid nitrogen and stored at −80°C before use. Total protein was extracted and used for further experiments. A, In-gel kinase assay using myelin basic protein (MBP) as a substrate. In-gel kinase assay was performed as described by Zhang and Klessig (1997). Briefly, the tissue was ground to fine powder in extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM sodium vanadate, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg mL^{-1} leupeptin, 5 μg mL^{-1} pepstatin A, 5 μg mL^{-1} aprotinin, and 10% [v/v] glycerol). The supernatant was collected after centrifugation at 16,000*g* at 4°C for 30 min. Total protein (15 μg) from each sample was separated in a 12% (w/v) SDS-PAGE containing MBP (0.25 mg mL^{-1}). After electrophoresis, the gel was washed four times for 20 min each at room temperature in the wash buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM sodium vanadate, 5 mM NaF, 0.5 mg mL^{-1} bovine serum albumin, and 0.1% [v/v] Triton X-100). To renature the kinases, the gel was incubated in renaturing buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM sodium vanadate, and 5 mM NaF) at 4°C overnight. The kinase reaction was performed by incubating the gel in 50 mL of kinase buffer (25 mM HEPES, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium vanadate, 200 nM ATP, and 50 μCi of γ-^{32}P-ATP; 3,000 Ci mmol^{-1}) for 1 h at room temperature. The gel was washed with several changes of 5% (w/v) trichloroacetic acid and 1% (w/v) NaPPi to remove the unincorporated γ-^{32}P-ATP. Autoradiography was performed using x-ray film (Kodak Biomax; Eastman-Kodak, Rochester, NY). B, Anti-AtPTP1 western-blot analysis. For raising anti-AtPTP1 antibodies, AtPTP1 protein (Xu et al., 1998) was used as an antigen to inject a rabbit at Cocalico Biological (Reamstown, PA). For western-blot analysis, total protein (30 μg) from each sample was separated in 12% (w/v) SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated in 5% (w/v) nonfat dry milk in Tris-buffered saline/Tween 20 (TBST; 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% [v/v] Tween 20) for 1 h at room temperature. Purified anti-AtPTP1 antibodies were used at a 1:500 dilution in TBST in the presence of 1% (w/v) nonfat dry milk. The bound antibody was detected by horseradish peroxide-conjugated anti-rabbit secondary antibodies (1:5,000 dilution in TBST + 1% [w/v] nonfat dry milk; Santa Cruz Biotechnology, Santa Cruz, CA) using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

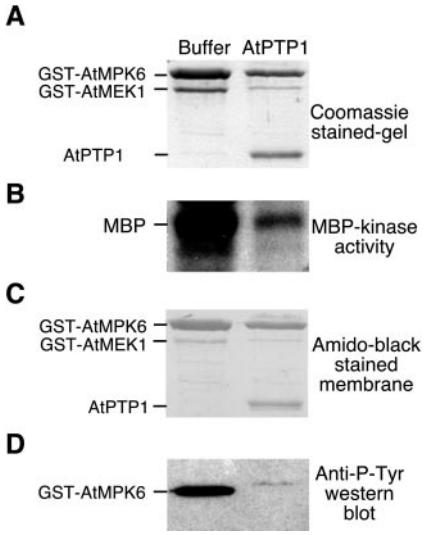

Figure 3. In vitro dephosphorylation and inactivation of AtMPK6 by AtPTP1. The coding sequences of AtMEK1 (an MAP kinase kinase; GenBank accession no. AAB97145), AtMPK6 (GenBank accession no. BAA04869), and AtPTP1 (Xu et al., 1998) were amplified by PCR using *pfu* polymerase and were cloned in frame with GST in pGEX-KG vector (Amersham Pharmacia). Fusion proteins (GST-AtMEK1, GST-AtMPK6, and GST-AtPTP1) were expressed and purified from *E. coli* as described previously (Gupta et al., 1998). The activation/inactivation of AtMPK6 and MBP kinase assay was performed as in earlier studies (Gupta et al., 1998; Huang et al., 2000). Briefly, GST-AtMEK1 and GST-AtMPK6 were mixed and incubated at 30°C for 30 min in the presence of 50 μM ATP in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM $MnCl_2$) to phosphorylate/activate the AtMPK6. The activated AtMPK6 was treated with AtPTP1 in the presence of 2 mM DTT for 30 min at 30°C followed by inactivation of AtPTP1 by treatment with 10 mM sodium vanadate for 15 min. AtMPK6 treated with buffer (lane buffer) and AtPTP1 (lane AtPTP1) was used for kinase assay at 30°C for 30 min with MBP (3 μg) and 10 μCi of γ-^{32}P-ATP (3,000 Ci mmol^{-1}) in the kinase buffer. Samples were resolved by SDS-PAGE, and autoradiography was performed using x-ray film (Kodak Biomax; Eastman-Kodak). A, Coomassie-stained gel showing the amount of GST-AtMPK6, GST-AtMEK1, and AtPTP1 used in the experiment. B, MBP-kinase activity of activated GST-AtMPK6 treated with buffer or AtPTP1. C, Amido-black stained membrane showing GST-AtMPK6 treated with buffer or AtPTP1 after transfer from the gel in A. D, Tyr-phosphorylation state of the activated GST-AtMPK6 treated with buffer or AtPTP1. Western blot was performed as described for Figure 2B. The membrane from C was probed with anti P-Tyr antibodies (PY99; Santa Cruz Biotechnology) at a dilution of 1:2,000. The bound antibody was detected by horseradish peroxide-conjugated anti-mouse (1:2,000 dilution) secondary antibodies (Santa Cruz Biotechnology) using an enhanced chemiluminescence kit (Amersham Pharmacia).

shown that H_2O_2 activates a 47-kD MAP kinase (Desikan et al., 2001; Yuasa et al., 2001), the kinetics of activation appeared to differ. H_2O_2 also activates ANP1, an MAP kinase kinase kinase. In Arabidopsis that initiates a phosphorylation cascade involving two stress MAP kinases, AtMPK3 and AtMPK6 (Kovtun et al., 2000). We also examined the level and stability of AtPTP1 protein in the total extracts by western-blot analysis. AtPTP1 antibodies were raised in a rabbit and were purified using affinity methods (Lin et al., 1996). Purified AtPTP1 antibodies recognized a single protein species at the predicted size of AtPTP1 protein. Consistent with the in vitro results, H_2O_2 treatment did not change the AtPTP1 protein level and stability in vivo (Fig. 2B). Studies have shown that the 47-kD MAP kinase activated by H_2O_2 is AtMPK6 (Desikan et al., 2001; Yuasa et al., 2001). To support the notion that AtPTP1 inactivation may be related to the activation of MAP kinase by H_2O_2, we expressed and purified glutathione *S*-transferase (GST)-AtPTP1, GST-AtMEK1, and GST-AtMPK6 from *Escherichia coli* as described previously (Gupta et al., 1998), and studied whether AtPTP1 can dephosphorylate and inactivate AtMPK6. As shown in Figure 3B, AtMEK1 (a MAP kinase kinase) activated AtMPK6 in a kinase assay using MBP as a substrate (Gupta et al., 1998). When the activated AtMPK6 was treated with AtPTP1, its activity was drastically reduced (Fig. 3B). It is clear from the data that AtPTP1 could inactivate AtMPK6 in vitro (Fig. 3B). Moreover, the inactivation of AtMPK6 is proportional to the extent of Tyr dephosphorylation in AtMPK6 (Fig. 3D).

In summary, these experiments show that AtPTP1 is reversibly inactivated by H_2O_2 without affecting the stability of AtPTP1 protein. AtPTP1 inactivation was strongly correlative to MAP kinase (AtMPK6) activation by H_2O_2, suggesting that AtPTP1 may represent a primary target for oxidative stress in plants.

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