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Short communication

Sulfhydryl-Related and Phenylpropanoid-Synthesizing Enzymes in *Arabidopsis thaliana* Leaves after Treatments with Hydrogen Peroxide, Heavy Metals, and Glyphosate

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Three-week grown *Arabidopsis thaliana* leaves were wounded by cutting whole leaves with a razor blade into pieces (about 3 mm × 3 mm), submerged in various solutions, and incubated in a growth chamber for 24 h. We measured and compared activities of several enzymes such as phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), thioredoxin, thioredoxin reductase, thioltransferase, glutathione reductase, and $NADP^+$-malate dehydrogenase. PAL activity was decreased in $HgCl_2$-, $CdCl_2$-, and glyphosate-treated leaf slices, and could not be detected after treatment with $CdCl_2$. TAL activity was found to be maximal in the $CdCl_2$-treated leaf slices. Activity of thioredoxin, a small protein known as a cofactor of ribonucleotide reductase and a regulator of photosynthesis, was significantly increased in the $CdCl_2$-treated leaf slices, while thioredoxin reductase activity was maximal in the $HgCl_2$-treated leaf slices. Thioltransferase and glutathione reductase activities were significantly decreased in the $HgCl_2$-treated leaf slices. $NADP^+$-malate dehydrogenase activity remained relatively constant after the chemical treatments. Our results strongly indicate that sulfhydryl-related and phenylpropanoid-synthesizing enzyme activities are affected by chemical treatments such as hydrogen peroxide, heavy metals, and glyphosate.

Keywords: *Arabidopsis thaliana*, Chemical treatment, Phenylalanine ammonia-lyase (PAL), Thioltransferase.

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Introduction

Recently, there has been great interest on the cellular responses to a variety of environmental stresses. However, the mechanisms of the early stress response prior to the expression of defense-related genes in plant cells are largely unknown. The location of the primary interaction between a stress and a cell may differ considerably depending on the nature of the stress. The literature includes studies on general responses to heat shock, radiation, glucose deprivation, heavy metals, oxidative stress, etc (Davies *et al.*, 1995). Phenylalanine ammonia-lyase (PAL), which plays key roles in plant development and in protection against environmental stress, is induced by various stress-related stimuli including wounding, heavy metals, light, phytotoxin, and phytochrome (Minamikawa and Uritani, 1965; Zucker, 1965; Heller *et al.*, 1979; Brödenfeldt and Mohr, 1988; Ohl *et al.*, 1990; Dubery and Smit, 1994; Smith *et al.*, 1994). Early light-inducible protein, a nuclear-encoded protein localized in the thylakoid membranes, is specifically induced by blue light and UV-A light in light-grown plants (Adamska *et al.*, 1992).

Thioredoxin was reported to be involved in the regeneration of proteins inactivated by oxidative stress in endothelial cells. It functions as an endogenous regeneration system for the inactivated proteins (Fernando *et al.*, 1992). Nicotinamide and trigonelline (N-methyl nicotinic acid) contents were increased in *Catharanthus roseus* tissue culture after exposure to 2,2′-azobis (2-amidinopropane) dihydrochloride or vanadylsulfate and in *Pisum sativum* leaves after exposure to UV-B radiation. Vanadylsulfate increased PAL activity and the contents of reduced and oxidized glutathione in *C. roseus* tissue culture (Berglund *et al.*, 1996). It has been hypothesized that nicotinamide and/or its metabolites may constitute a link between various types of stresses, especially stress

causing DNA strand breaks and the induction of defense metabolism in eukaryotic cells.

In this communication, we report the changes in sulfhydryl-related and phenylpropanoid-synthesizing enzyme activities in *Arabidopsis thaliana* leaves after treatments with hydrogen peroxide, heavy metals, and glyphosate. Enzyme activities tested include (1) thioredoxin, thioltransferase, glutathione reductase, and thioredoxin reductase involved in thiol/disulfide exchange reactions; (2) PAL and TAL involved in the biosynthesis of phenylpropanoids; and (3) $NADP^+$-malate dehydrogenase.

Materials and Methods

Chemicals Bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (yeast), NADPH, Tris, 5,5′-dithio-2-nitrobenzoic acid (DTNB), acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate, EDTA, L-phenylalanine, L-tyrosine, oxaloacetate, and Coomassie Brilliant Blue R-250 were obtained from Sigma Chemical Co. (St. Louis, USA). 2-Hydroxyethyl disulfide (HED) was purchased from Aldrich Chemical Co. (Milwaukee, USA). Vermiculite, perlite, and peat moss were obtained from a gardening shop in Chuncheon, Korea. *E. coli* thioredoxin reductase was kindly provided by Prof. James A. Fuchs, University of Minnesota, St. Paul, Minnesota, USA. All other chemicals and reagents used were of the highest grade commercially available.

Growth of plants Seeds of *Arabidopsis thaliana* ecotype Columbia were kindly provided by Prof. Hong-Gil Nam, Pohang University of Science and Technology, Korea. Seeds were cultivated in soil, in a 1:1:1 mixture of vermiculite, perlite, and peat moss. Cultivation conditions were set to 26°C and 60% humidity in a growth chamber.

Wounding and chemical treatment Three-wk-old *Arabidopsis* leaves were taken and wounded by cutting whole leaves with a razor blade into pieces (about 3 mm × 3 mm). The sliced leaves were submerged in solutions containing H_2O_2 (1 mM), $HgCl_2$ (0.1 mM), $CdCl_2$ (0.25 mM), or glyphosate (0.05 mM) for 24 h in a growth chamber. Concentrations of the chemicals used were determined in reference to those in the previous literatures.

Preparation of leaf extract The *Arabidopsis thalinana* leaves, wounded or treated with chemicals, were washed very cleanly and were ground up with buffer A (50 mM Tris·HCl containing 1 mM EDTA, pH 7.5) in a mortar containing sea sand. The mixture was then clarified by centrifugation (10,000 × *g*, 10 min) at 4°C and the supernatant was saved for enzyme assays.

Determination of enzyme activities

Thioltransferase activity: Thioltransferase catalyzes the reduction of certain disulfides in the presence of glutathione and thus has GSH-disulfide-transhydrogenase activity (Cho *et al.*, 1998). In the present study, 2-hydroxyethyl disulfide was used as a substrate. Two quartz semi-microcuvettes with a 1-cm light path contained 500 μl of mixture at room temperature. To both cuvettes were added 1.5 mM 2-hydroxyethyl disulfide, 100 μg/ml bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris·HCl (pH 8.0), 2 mM EDTA. The absorbance at 340 nm was recorded for 2 min to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between glutathione and 2-hydroxyethyl disulfide. Leaf extract was added to the sample cuvette and an equal volume of buffer A was added to the reference cuvette. The decrease in absorbance was then recorded for a few min. The result was calculated as ΔA_{340}/min.

Phenylalanine ammonia-lyase activity: Phenylalanine ammonia-lyase activity was measured by a modification of the spectrophotometric assay as described previously (Lim *et al.*, 1998). The reaction mixture contained 50 mM Tris·HCl (pH 9.0), 2 mM L-phenylalanine, and leaf extract in a total volume of 1.5 ml. The assay was carried out at 30°C and the reaction was stopped by the addition of 1 ml of 2 N HCl. The *t*-cinnamic acid formed was extracted into 2 ml of toluene by vortexing for 10 s and centrifuging at 1500 × *g* for 10 min. The absorbance at 290 nm of *t*-cinnamic acid recovered in the toluene phase was measured using toluene as a blank.

Glutathione reductase activity: The oxidation of NADPH was followed spectrophotometrically at 340 nm (Carlberg and Mannervik, 1985). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 0.1 mM NADPH, and 1 mM GSSG in a total volume of 1 ml. The reaction was initiated by the addition of the leaf extract to the cuvette and the decrease in absorbance at 340 nm was followed. The glutathione reductase activity was expressed as ΔA_{340}/min.

Thioredoxin activity: Thioredoxin catalyzes the NADPH-dependent reduction of the disulfide bond in DTNB (Luthman and Holmgren, 1982). The assay mixture contained 100 mM Tris·HCl (pH 8.0), 2 mM DTNB, and 0.24 mM NADPH in a volume of 1.0 ml. Leaf extract was added into the sample cuvette, whereas buffer A was added into the reference cuvette. The reaction was initiated by adding thioredoxin reductase. An increase in absorbance at 412 nm was directly monitored using a spectrophotometer. Thioredoxin activity was expressed as ΔA_{412}/min.

Tyrosine ammonia-lyase activity: The deamination of tyrosine by tyrosine ammonia-lyase was monitored as described previously (Abell and Shen, 1987). For tyrosine ammonia-lyase activity, 2.5 ml of 12 mM L-tyrosine in 0.1 M Tris·HCl buffer (pH 8.5) was added as a substrate to leaf extract. The blank did not contain L-tyrosine. The increase in absorbance at 333 nm was recorded spectrophotometrically after incubation. The activity was expressed as ΔA_{333}/min.

Thioredoxin reductase activity: The activity of thioredoxin reductase was assayed in the presence of thioredoxin as a direct substrate (Lim and Lim, 1995). The reaction mixture contained 0.1 M Tris·HCl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, 0.1 mg/ml BSA, *E. coli* thioredoxin, and leaf extract. The reaction was started by adding leaf extract and the absorbance increase at 412 nm was recorded.

Malate dehydrogenase activity: $NADP^+$-malate dehydrogenase activity was measured as described previously (Jacquot *et al.*, 1995). The reaction mixture contained 0.1 M Tris·HCl buffer (pH 8.0), 0.75 mM oxaloacetate, and 0.15 mM NADPH in a volume of 1 ml. The reaction was initiated by the addition of leaf extract. It was followed spectrophotometrically by the decrease of absorbance at 340 nm. The activity was expressed as ΔA_{340}/min.

Protein determination The protein content in leaf extract was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Results and Discussion

Treatment with hydrogen peroxide, heavy metals, or glyphosate on growing plant leaves causes variations in the activities of various enzymes, as already reported (Bednar *et al.*, 1989; Lamb *et al.*, 1998). According to the results of Herouart *et al.* (1996), exposure of a sublethal concentration of hydrogen peroxide to *Rhizobium meliloti* leaves enhances catalase activity (100-fold) and also increases survival to subsequent hydrogen peroxide exposure at higher concentrations. Hydrogen peroxide is a representative chemical frequently used in oxidative stress experiments. Chalcone isomerase from soybean is inactivated by stoichiometric amounts of *p*-mercuribenzoate or $HgCl_2$, indicating that only half-cystine residue is located in its active site (Bednar *et al.*, 1989). Glutathione is essential for protecting plants from a range of environmental stresses, including heavy metals where it acts as a precursor for the synthesis of phytochelatins. *Arabidopsis thaliana* plants were reported to actively synthesize glutathione upon exposure to cadmium, due to the increased level of glutathione synthetase (Wang and Oliver, 1996). Glyphosate is a herbicide extensively used worldwide which acts as an inhibitor of 5′-enolpyruvylshikimate-3 phosphate synthase, which is involved in an essential step in the shikimate pathway common to aromatic acid biosynthesis, and has broad spectrum herbicidal activity against a wide range of annual and perennial weeds. It was found to act as a strong inhibitor of cytochrome P450 (Lamb *et al.*, 1998). In this investigation, we investigated variations in activity of sulfhydryl repair enzymes and phenylpropanoid-synthesizing enzymes after the treatments with hydrogen peroxide, heavy metals ($Hg^{2+}$and Cd^{2+}), and glyphosate. Chemical treatments were performed using the wounded leaves of *Arabidopsis thaliana.*

Wounding effect Mechanical damage to leaf tissue causes variations in several enzymes such as 1-aminocyclopropane-1-carboxylate oxidase (Bouquin *et al.*, 1997) and omega-3 fatty acid desaturase (Hamada *et al.*, 1996). In the wounded leaves of *Arabidopsis thaliana*, the activity of PAL was increased significantly (Table 1). This fact was consistent with previous identified results (Minamikawa and Uritani, 1995). Thioredoxin and TAL activities were also increased, whereas thioltransferase, thioredoxin reductase, and glutathione reductase were decreased. From these results, wounding itself could affect the activities of sulfhydryl-related enzymes and phenylpropanoid synthesizing enzymes in the leaves of *Arabidopsis thaliana.* Increased PAL and TAL activities may induce the syntheses of phenylpropanoid

Table 1. Variations in specific activity of sulfhydryl-related and phenylpropanoid-synthesizing enzymes of *Arabidopsis thaliana* leaves after wounding.

Enzymes	Specific Activities	
	Control	Wounding
Sulfhydryl-related enzymes		
Thioredoxin[a]	0.043 ± 0.002 (100)	0.055 ± 0.003 (128)
Thioredoxin reductase[b]	0.054 ± 0.003 (100)	0.033 ± 0.002 (61)
Thioltransferase[c]	1.12 ± 0.06 (100)	0.93 ± 0.05 (83)
Glutathione reductase[d]	0.29 ± 0.01 (100)	0.22 ± 0.01 (76)
Phenylpropanoid-synthesizing enzymes		
L-Phenylalanine ammonia-lyase[e]	0.0017 ± 0.0002 (100)	0.0037 ± 0.0005 (220)
L-Tyrosine ammonia-lyase[f]	0.053 ± 0.003 (100)	0.072 ± 0.004 (136)
$NADP^+$-malate dehydrogenase[g]	0.043 ± 0.002 (100)	0.059 ± 0.003 (137)

[a] Specific activity of thioredoxin was expressed as ΔA_{412}/min/mg protein.
[b] Specific activity of thioredoxin reductase was expressed as ΔA_{412}/min/mg protein.
[c] Specific activity of thioltransferase was expressed as ΔA_{340}/min/mg protein.
[d] Specific activity of glutathione reductase was expressed as ΔA_{340}/min/mg protein.
[e] Specific activity of phenylalanine ammonia-lyase was expressed as ΔA_{290}/min/mg protein.
[f] Specific activity of tyrosine ammonia-lyase was expressed as ΔA_{333}/min/mg protein.
[g] Specific activity of $NADP^+$-malate dehydrogenase was expressed as ΔA_{340}/min/mg protein

compounds in the wounded leaves, the reason of which remains unclear. However, phenylpropanoid compounds might be involved in the defense mechanism of the wounded leaves.

Sulfhydryl-related enzyme activities In plant cells, thioredoxin is known to act as an important regulator of photosynthesis. The regulation is carried out by modulating several enzymes involved in the Calvin cycle. Treatment of hydrogen peroxide on the wounded leaves of *Arabidopsis thaliana* significantly decreased thioredoxin activity whereas cadmium chloride treatment greatly increased the activity (Fig. 1). Treatments of mercuric chloride and glyphosate did not give significant changes in thioredoxin activity. However, higher concentration (0.2 mM) of mercuric chloride gave a large decrease in thioredoxin activity (data not shown). This might result from the fact that the active-site thiol group in thioredoxin could be inactivated by mercuric chloride.

Thioltransferase catalyzes the reversible thiol-disulfide interchange reactions. The enzyme has a major role in maintaining intracellular thiol in the reduced state and functions in this capacity by coupling to glutathione and glutathione reductase. Thioltransferase also has a role in cellular regulation by catalyzing the reversible modification of proteins by thiol-disulfide interchange. Treatment with heavy metals, mercuric chloride and cadmium chloride, on the wounded *Arabidopsis thaliana* leaves caused a significant decrease in the activity of thioltransferase (Fig. 2). A higher concentration (0.2 mM) of mercuric chloride completely inactivated the thioltransferase activity (data not shown). Considering that cadmium increases the synthesis of glutathione (Wang and Oliver, 1996), a decrease in thioltransferase activity using glutathione as a cofactor by cadmium treatment seems to be affected in a different manner. However, this inactivation could also be due to the interaction between the active site and mercuric ions. Treatments with hydrogen peroxide or glyphosate gave no significant changes in thioltransferase activity.

Thioredoxin reductase reduces the oxidized thioredoxin in various types of cells. Thioredoxin is also reduced by ferredoxin-thioredoxin reductase in plant cells. Thioredoxin reductase activity was greatly enhanced by the treatment of mercuric chloride, whereas it remained relatively constant after treatments with hydrogen peroxide, cadmium chloride, and glyphosate (Fig. 3). Although thioredoxin reductase also contains two half-cysteine residues in the active site, it was not affected by mercuric chloride. A lower concentration (0.2 mM) of cadmium chloride caused a significant increase in thioredoxin reductase activity (data not shown). This suggests that the concentration of heavy metals is an important factor in modulating enzyme activities.

Glutathione reductase is a flavoprotein catalyzing the NADPH-dependent reduction of glutathione disulfide to glutathione. The reaction is essential for the maintenance of glutathione levels. Glutathione reductase activity in the

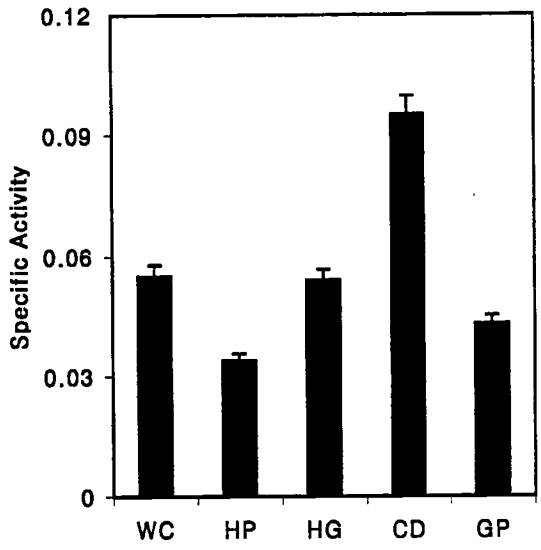

Fig 1. Variations in specific activities of thioredoxin in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{412}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

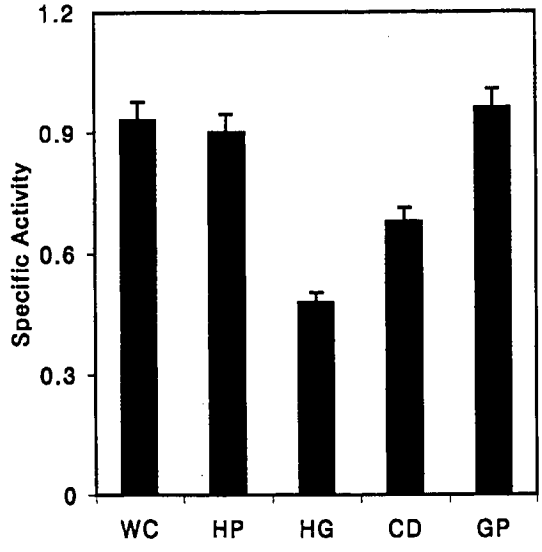

Fig 2. Variations in specific activities of thioltransferase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{340}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

wounded leaves of *Arabidopsis thaliana* was greatly decreased after the treatment of mercuric chloride (Fig. 4). However, cadmium chloride gave a slightly higher value, whereas glyphosate treatment gave a slightly lower value. Previously, glutathione synthesis was reported to be increased by cadmium treatment.

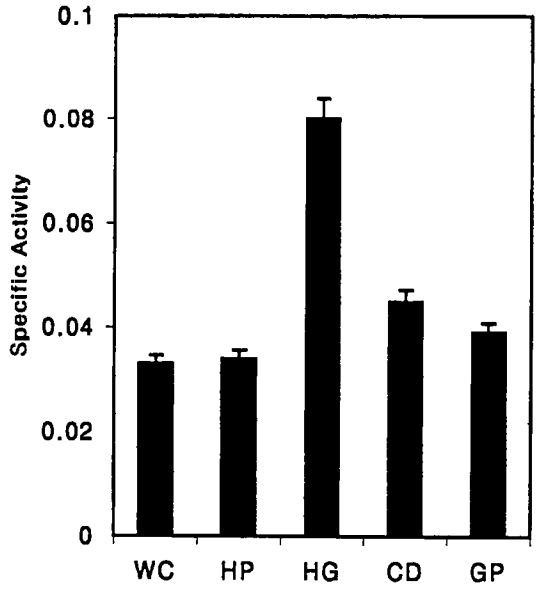

Fig 3. Variations in specific activities of thioredoxin reductase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{412}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

Enzymes involved in the phenylpropanoid pathway The two enzymes which are involved in phenylpropanoid metabolism, PAL and TAL, are highly regulated by events taking place during various plant development stages. PAL and TAL convert L-phenylalanine and L-tyrosine to ammonia and *t*-cinnamic acid and *p*-coumaric acid, respectively, which are further modified in phenylpropanoid metabolism to precursors used in secondary pathways producing lignin, flavonoids, anthocyanins, phytoalexins, and tannins. PAL is known to be involved in the defense mechanism against environmental stresses. Variations in PAL activity after chemical treatment are shown in Fig. 5. Treatment with cadmium chloride gave a complete reduction in PAL activity in the wounded leaves of *Arabidopsis thaliana*. Its lower concentration (0.2 mM) caused less reduction in PAL activity (data not shown). Treatments with mercuric chloride and glyphosate also caused a significant reduction in PAL activity, whereas hydrogen peroxide had no effect on PAL activity. In the case of a higher concentration (0.2 mM) of mercuric chloride, PAL activity was not detected (data not shown). Reduction of PAL activity by glyphosate treatment attracts an interest.

TAL activity in the wounded leaves after chemical treatment was shown in Fig. 6. Treatment with mercuric chloride or glyphosate made a significant reduction in TAL

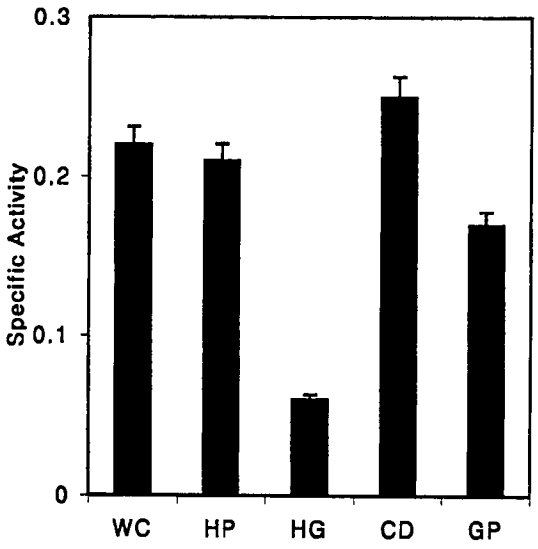

Fig 4. Variations in specific activities of glutathione reductase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{340}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

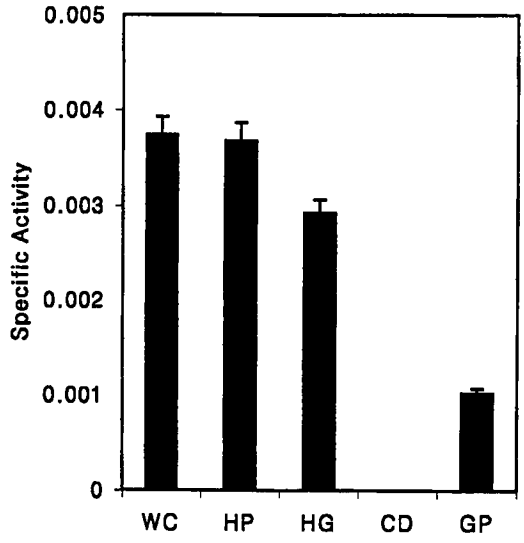

Fig 5. Variations in specific activities of L-phenylalanine ammonia-lyase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{290}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

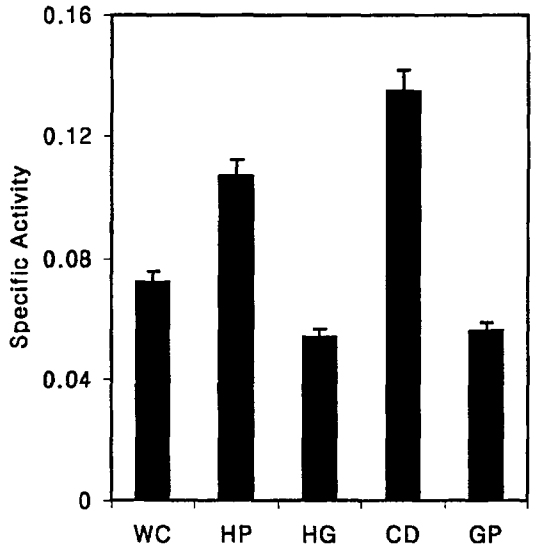

Fig 6. Variations in specific activities of L-tyrosine ammonia-lyase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{333}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

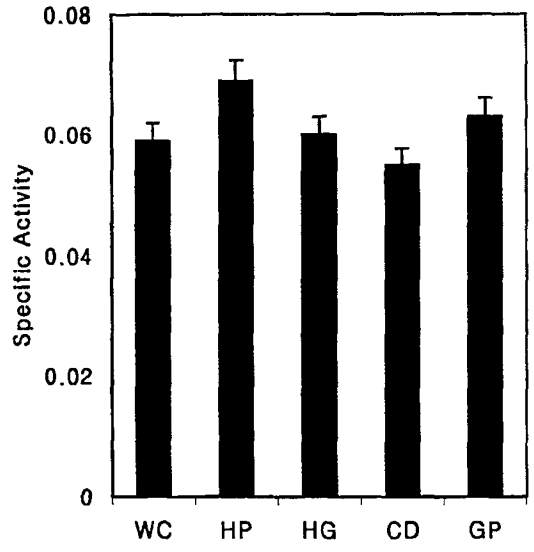

Fig 7. Variations in specific activities of $NADP^+$-malate dehydrogenase in the wounded leaves of *Arabidopsis thaliana* after treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate. Its specific activity was expressed as ΔA_{340}/min/mg protein. WC, wounding control; HP, hydrogen peroxide (1 mM); HG, mercuric chloride (0.1 mM); CD, cadmium chloride (0.25 mM); GP, glyphosate (0.05 mM).

activity. However, cadmium chloride treatment gave a significant increase in TAL activity. A higher concentration (0.2 mM) of mercuric chloride gave more significant reduction in TAL activity (data not shown). Our results showed treatments with mercuric chloride and glyphosate in the wounded leaves inactivated both PAL and TAL activities. It indicates that they may inhibit the synthesis of phenylpropanoid compounds.

$NADP^+$-malate dehydrogenase $NADP^+$-specific malate dehydrogenase, which is known to be activated by m-type thioredoxin (Ruelland *et al.*, 1997), catalyzes the reduction of oxaloacetate using NADPH as a reductant. A high activity of the enzyme is detected in C_4 variants in which malate is the principal short-term product of CO_2 assimilation and is located in the chloroplasts of mesophyll cells. In treatments with hydrogen peroxide, heavy metals (Hg^{2+} and Cd^{2+}), and glyphosate, no significant variations were detected in the activity of $NADP^+$-malate dehydrogenase (Fig. 7).

In this investigation, we measured activities of sulfhydryl-related enzymes and PAL and TAL in the wounded leaves of *Arabidopsis thaliana* after treatment with hydrogen peroxide, heavy metals, and glyphosate. Each chemical induced a specific variation in the enzyme activities tested. Therefore, it might be assumed that sulfhydryl-related enzymes and PAL and TAL are closely related with environmental stresses. Although thioredoxin is involved in the regeneration of animal proteins damaged by oxidative stress (Fernando *et al.*, 1992), its activity was decreased after treatment with hydrogen peroxide in the leaves of *Arabidopsis thaliana.* However, it did not give any change in thioltransferase activity. Further study should be done to elucidate the relationship between sulfhydryl-related enzymes and PAL and TAL and chemical stress.

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