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Changes in the Isozyme Composition of Antioxidant Enzymes in Response to Aminotriazole in Leaves of *Arabidopsis thaliana*

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The changes in isozyme profiles of catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) during severe deactivation of total CAT activity by aminotriazole (AT) treatment were investigated in the leaves of *Arabidopsis thaliana* (Columbia ecotype) in relation to H_2O_2-mediated oxidative stress. In spite of striking deactivation of total CAT activity by 0.1 mM AT, there were no significant differences in H_2O_2 levels or total leaf soluble protein contents including a Rubisco in both the control and AT-treated leaves. On the other hand, one specific protein band (molecular mass, 66 kD) was observed on the SDS-gel from leaf soluble proteins whose staining intensity was strikingly enhanced by AT treatment for 6 h. However, this band disappeared at 12 h. In the native-gel assays of CAT, POD, APX and GR isozymes, AT remarkably inhibited the expression of the CAT1 isozyme with no effects on CAT2 and CAT3, and generally had no effect on POD isozyme profiles. However, AT stimulated the intensity of activities of pre-existing APX1 and GR1 isozymes. In particular, it induced a new synthesis of one GR isozyme. Therefore, these results collectively suggest that a striking deactivation of total CAT activity by AT in *A. thaliana* leaves largely results from the suppression of CAT1 isozyme, and that APX1, GR1, and a newly synthesized GR isozyme could complement the role of CAT1 to metabolize H_2O_2 into non-toxic water.

Keywords: aminotriazole, antioxidant enzyme, *Arabidopsis thaliana*, H_2O_2, isozyme

Since the removal of H_2O_2 in plant cells is carried out exclusively by catalase (CAT), and the herbicide aminotriazole (AT) is known to be a specific CAT inhibitor (Tsaftaris and Scandalios, 1981; Harvir, 1992), a part of the action of AT may be associated with hydrogen peroxide-linked toxicity. Indeed, it has been used as a tool to artificially inhibit CAT activity and to initiate the accumulation of oxidative stress in plants (Ferguson and Dunning, 1986; Prasad et al., 1994; Prasad, 1997). During plant damages from stress, the role of activated oxygen species (AOS) including superoxides and H_2O_2 is well indicated by the increased accumulation of AOS in plant tissues (Allen, 1995), and the importance of antioxidant enzymes to scavenge AOS is generally emphasized in preventing oxidative stress (Anderson et al., 1995; Rao et al., 1996; Donahue et al., 1997; Prasad, 1997). In recent years, many studies have been initiated to enhance plant tolerance to oxidative stress by modifying the plant antioxidant defense system (Sen Gupta et al., 1993; Foyer et al., 1994; Allen, 1995). It has been known that CAT, peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) are the major constituents of the plant antioxidant enzyme system,which operate by scavenging AOS (Klapheck et al., 1990; Anderson et al., 1995; Rao et al., 1996). Plants are also reported to possess different isozymes of CAT, POD, APX, and GR (Mittler and Zilinskas, 1993; Anderson et al., 1995; Rao et al., 1995; Prasad, 1997). In addition, it is believed that oxidative stress-induced changes in the total activities of antioxidant enzymes could be due to changes in their protein content and/or the synthesis of new isozymes (Edward et al., 1994; Rao et al., 1995). Edward et al. (1994) have suggested that the synthesis of new isozymes of antioxidant enzymes may be more beneficial for the metabolism of oxygen radicals than the enhancement of the activities of existing antioxidant enzymes. In our previous study (Kang et al., 1998), we observed the enhanced activities of total APX and GR are concomitant with severe CAT deactivation in *Arabidopsis thaliana* leaves treated with 0.1mM AT.

Therefore, we have attempted: (a) to determine whether the AT-induced change in total activities of CAT, POD, APX, and GR reflect changes in their isozyme compositions and (b) to establish which isozymes are more important for providing a tolerance to AT in *A. thaliana* leaves.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *A. thaliana* (Columbia ecotype) were sown and germinated in plastic pots (115× 115×100 mm) containing commercial soils (Ball Seed Co., Canada) moistened with distilled water. After sowing, the pots were covered with a transparent plastic film to prevent desiccation for the first 4 days. The environmental conditions in the growth chamber containing the germination pots were 24/21°C (day/night) temperature, 70% relative humidity, and a 7000 lux of light intensity with an 18 h photoperiod. From the 5th day, the seeding plants were watered with half-strength Hoagland mineral solution on alternate days. Then, twelve-day-old plants were treated with 0.1 mM 3-amino-1,2,4-triazole (AT) and grown further for 3 days in the same growth chamber. Control plants were grown without AT treatment.

Preparation of Cell-Free Extracts

Cell-free leaf extracts were obtained as described below for the total CAT activity assay, SDS-PAGE of foliar proteins, and native PAGE of CAT, POD, APX, and GR isozymes. 10 plant leaves were homogenized with 4 mL of a 0.1 M potassium phosphate (pH 7.5) buffer containing 2 mM EDTA and 1% PVPP. The supernatant, recovered at 4°C by centrifugation (20,000*g*, 20 min), was used for the total CAT activity assay. For the leaf extracts of SDS-PAGE analysis, 0.1 M Tris-HCl (pH 7.5) containing 1% PVPP was used as an extraction medium. Insoluble material was removed by centrifugation (20,000*g*, 20 min). Since maintenance of constant electrophoretic mobility of CAT and GR isozymes on native gels was found to require the presence of DTT (Anderson et al.,1995), 120 leaves were ground in 1.5 mL of a 60 mM Tris-HCl (pH 6.9) buffer containing 1 mM EDTA, 10% PVPP, and 10 mM DTT for CAT isozymes, and the other 120 leaves were homogenized in 1.5 mL of a 0.1 M sodium phosphate (pH 7.0) buffer containing 1 mM EDTA, 1% PVPP, and 10 mM DTT for GR isozyme extracts. For the POD and APX isozyme extracts, 120 leaves were also homogenized in 1.5 mL of a 0.1 M sodium phosphate (pH 7.0) buffer containing 5 mM ascorbate, 1 mM EDTA, and 1% PVPP. After removal of the insoluble materials by centrifugation (20,000*g*, 20 min), all extracts were immediately stored at –80°C until analysis. However, samples for CAT and APX gels were made fresh right before electrophoresis. Protein content in extracts was quantified according to Lowry et al. (1951).

Assay of Total CAT Activity

Total CAT activity was determined spectrophotometrically by monitoring the decline in A_{240} due to H_2O_2 decomposition ($\varepsilon = 39.4\ M^{-1}cm^{-1}$). The 3 mL reaction mixture contained 0.1 M potassium phosphate (pH 7.0), 10 mM H_2O_2, and enzyme extract. The reaction was initiated by adding 10 mM H_2O_2 (Rao et al., 1996).

SDS-PAGE of Foliar Proteins and Rubisco Protein Determination

Cell-free leaf extracts were denatured by adding of an equal volume of a sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.004% BPB) and heating at 95°C for 3 min. After cooling, equal amounts of protein (50 μg) from extracts of different treatments were loaded onto slab gels containing a 12% separating polyacrylamide gel with a 4% stacking gel, using the buffer system of Laemmli (1970). Proteins were visualized by staining the gels with 0.25% Coomassie brilliant blue R-250 (CBB-R) in 45% methanol and 10% acetic acid at room temperature for 1 h and destained with succesive changes of 20% methanol and 7% acetic acid. For determination of the rubisco protein level, the CBB-R dye bound to rubisco bands on the gel was eluted with formamide, and the absorbance of the resultant solution was measured spectrophotometrically according to Amane et al. (1986). The stained bands were cut out of the gels with a razor blade and eluted in 1.0 mL of formamide in amber vials at 50°C for 5 h with gentle shaking. The absorbance of the formamide-CBB-R solutions was read at 595 nm. The relative amounts of rubisco proteins were estimated as their absorbance values.

Native PAGE and Activity Staining

Equal amounts of protein from leaf extracts were subjected to native PAGE using the discontinuous buffer system of Laemmli (1970), except that SDS was omitted. CAT isozymes were separated on the 7% separating polyacrylamide gel with a 4% stacking gel at 100 V for 6 h at 4°C (Kunce and Trelese, 1986). The loading sample contained 60 mM Tris-HCl (pH 6.9), 10% glycerol, 0.001% BPB and CAT extract (30

μg protein). After electrophoresis, the gels were negatively stained for CAT activity as described by Woodbury et al. (1971). The gels were incubated in 3.27 mM H_2O_2 for 25 min, rinsed in water, and soaked in a solution of 1% ferric chloride-potassium ferricyanide (III). POD isozymes were separated on the 10% separating gel with a 4% stacking gel at 100 V for 7 h at 4°C. The loading samples contained 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and POD extract (120 μg protein). Staining of POD isozymes was performed by incubating the gels in 50 mM sodium acetate buffer (pH 4.5) containing 2 mM benzidine for 10 min. The reaction was initiated by adding 3 mM H_2O_2 and was allowed to continue for 20 min. Thereafter, the reaction was stopped by a brief wash in deionized water and immediately photographed (Rao et al., 1996).

GR isozymes were separated on the 7% separating gel at 100 V for 3 h at 4°C. The loading samples contained 125 mM Tris-HCl (pH 6.9), 5% glycerol, 0.002% BPB, and GR extract (100 μg protein). GR activity was detected by incubating gels in a solution of 0.25 M Tris-HCl (pH 7.8) containing 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol, and 3.6 mM GSSG in darkness for 1 h. Duplicate gels were assayed for GSSG-specific GR activity, one with and one without GSSG (Anderson et al., 1995). For separation of APX isozymes, the samples containing 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and APX extract (120 μg protein) were subjected to native PAGE (10% separation gel, 4% stacking gel) as described by Laemmli (1970) except that SDS was omitted, and the tank buffer contained 2 mM ascorbate. Electrophoresis was performed with a constant current of 10 mA for 6 h at 4°C. The gels were prerun for 30 min to allow the 2 mM ascorbate to enter the gel prior to the application of the samples (Mittler and Zilinskas, 1993). After electrophoresis, the gels were equilibrated with a 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were then incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed with a 50 mM potassium phosphate buffer (pH 7.0) for 1 min and soaked in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium. The APX activity was observed as an achromatic band on a purple-blue background.

Measurement of H_2O_2 Content

For the measurement of H_2O_2 content, 15 leaves were homogenized by mortar and pestle using 3 mL of a 100 mM Na-phosphate buffer (pH 6.8). The homogenate was centrifuged at 18,000*g* for 20 min and the supernatant was used for assay. Determination of H_2O_2 content was performed basically according to Bernt and Bergmeryer (1974) using a peroxidase enzyme (Sigma Co.). An aliquot of 0.5 mL of supernatant was mixed with 2.5 mL of peroxide reagent (83 mM sodium phosphate, pH 7.0, 0.005% o-dianisidine, 40 μg peroxidase/mL) and then incubated at 30°C for 10 min. The reaction was stopped by adding 0.5 mL of 1 N perchloric acid and centrifuged at 3,000*g* for 3 min. The resultant supernatant was read at 436 nm, and its absorbance was compared to that of H_2O_2 standard.

RESULTS AND DISCUSSION

AT Application and Changes of Total CAT Activities, H_2O_2 Levels and Soluble Protein Profiles

One of the major roles of activated oxygen species (AOS), including the superoxide anions and hydrogen peroxide, is to modify the structure of cellular proteins (Pacifici and Davis, 1990), thus enhancing their susceptibility to proteolysis (Stadtman, 1990). In particular, oxidation of [-SH] radical-containing proteins is common under a variety of oxidative stress conditions (Penarrubia and Moreno, 1990). Recently, Desimone et al. (1996) reported the activated oxygen-mediated denaturation of rubisco proteins and proteolysis of its large subunits in barley chloroplasts grown under herbicide treatments. Since AT is expected to generate AOS, including H_2O_2 in cells, we investigated an impact of AT on the content and profiles of soluble proteins with parallel assays of H_2O_2 level and total CAT activity in growing leaves of *A. thaliana* plants. When 12-day-old seedings were treated with 0.1 mM AT for 3 days, severe CAT deactivation (*ca.* 61% reduction) was found after 1 day of treatment, and the deactivation continued for 3 days (Table 1). Unexpectedly, there was, however, no significant difference in either the H_2O_2 levels or the soluble protein contents in both the control and AT-treated leaves (Table 1).

Also, no significant changes were observed in the amounts of large and small subunits of rubisco in AT-

Table 1. Effects of 0.1 mM aminotriazole (AT) on the developmental change of total catalase activity, H_2O_2 and soluble protein contents in *A. thaliana* leaves. An AT treatment was initiated when seedlings were 12-day-old, and it continued for 3days. Measurements were performed 1d, 2d and 3d after treatment of seedling with AT.

Treatment (day)		Catalase (mM H_2O_2 min^{-1} mg^{-1} protein)	H_2O_2 ($\mu g\ g^{-1}$ fr. wt.)	Soluble protein ($mg\ g^{-1}$ fr. wt.)
1d	Control	150.1±18.0	0.033±0.003	4.67±0.24
	AT	58.5±28.4	0.041±0.001	5.14±0.69
2d	Control	139.2±36.3	0.024±0.004	3.94±0.58
	AT	64.8±14.6	0.029±0.009	3.43±0.47
3d	Control	148.3±19.8	0.021±0.001	4.35±0.16
	AT	44.4± 5.7	0.022±0.002	4.56±0.45

Data are the means ± SE (n = 3)

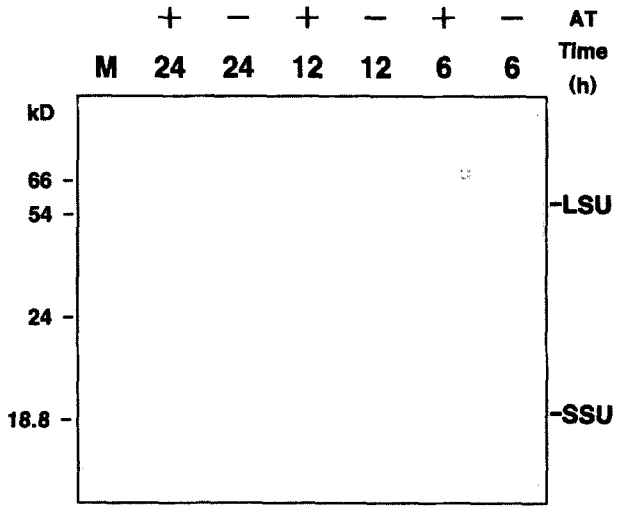

Figure 1. SDS-PAGE of leaf soluble proteins of *A. thaliana* plants grown in the presence (+) or absence (–) of 0.1 mM AT for 24 h. An AT treatment was initiated when seedling plants were 12-day-old. The numbers in each lane indicate the time (h) of AT treatment and the lane "M" represents the standard molecular marker proteins. The abbreviations of "LSU" and "SSU" denote the large and small subunits of Rubisco, respectively. The arrow ("←") shows a protein band (molecular mass, 66 kD) whose staining intensity was strikingly enhanced by AT treatment for 6 h.

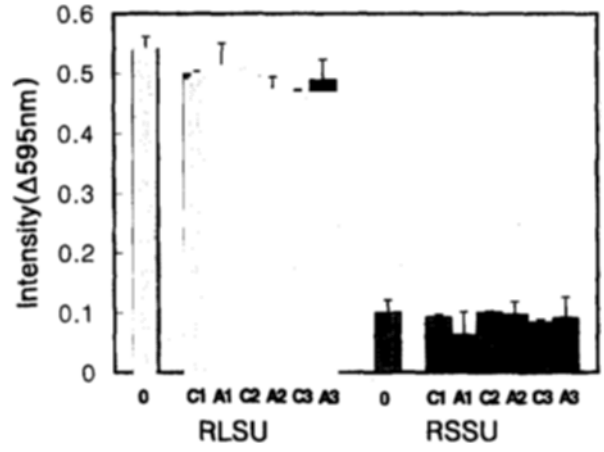

Figure 2. Staining intensities of the large (RLSU) and small (RSSU) subunits of Rubisco in leaves of *A. thaliana* grown in the presence (A) or absence (C) of 0.1 mM AT. The numbers in the X-axis indicate the time of AT treatment. Error bars represent SE.

treated leaves compared to those of control leaves (Fig. 2). In addition, the total soluble protein profile including Rubisco in control leaves was also similar to those in AT-treated leaves (Figs. 1 and 2). These results indicate that AT-induced CAT deactivation in leaves of *A. thaliana* does not necessarily imply an automatic oxidative damage to cellular proteins. Therefore, it is likely that other antioxidant enzymes in the cell could contribute in a protective process against AT toxicity. Interestingly, one protein band of unknown identity (molecular mass, 66kD) was distinctively expressed in AT-treated leaves at 6 h, and it disappeared again at 12 h (Fig. 1). This means that there is a possibility that some changes in the cellular metabolic events connected to CAT deactivation was caused in response to AT treatment in *A. thaliana* leaves. Furthermore, our previous study (Kang et al., 1998) showed that an increase in total activities of APX and GR is concomitant with severe CAT deactivation in the leaves of *A. thaliana* with 0.1 mM AT application. Therefore, we first attempted to determine whether the AT-induced changes in total activities of CAT, APX, and GR reflect changes in their isozyme compositions in leaves of *A. thaliana* plants.

CAT Isozymes and Specificity of AT for Their Inhibition

To identify any changes in CAT isozyme profiles affected by AT treatment, leaf extracts of *A. thaliana*

were subjected to native PAGE and monitored for CAT activity (Fig. 3). We observed a severe decline in total CAT activity in the leaves of *A. thaliana* under AT treatment (Table 1). With such a result, Figure 3 illustrates the presence of three CAT isozymes in leaves. According to the CAT isozymes profiles, the faster-migrating CAT1 isozyme contributed prominently to the total CAT activity in the leaves, and a decrease of total CAT activity by AT appeared to be primarily due to an striking decline in CAT1. The activity of the slow-migrating CAT2 isozyme became lower than that of CAT1. However, the activity was not shown to be inhibited by AT. CAT in maize seedlings are reported to be present as four isozymes (Scandalios et al., 1984): two of them (CAT1 and CAT3) are present only in dark-grown shoots, and one isozyme (CAT2) is present only in photosynthetic tissues (Anderson et al., 1995). Also, Anderson et al. (1995) and Prasad (1997) have found that the peroxisomal CAT1 migrates faster than the mitochondrial CAT3 in native gels. Chandlee et al. (1983) suggested that AT inhibits the peroxisomal CAT1 and CAT2 isozymes more prominently than the mitochodrial CAT3. Therefore, it might appear likely that AT in *A. thaliana* also inhibits total CAT activities through an inactivation of peroxisomal CAT1 isozyme. The intensity of the slowest-migrating CAT3 was very weak among them; thus its activity was not well recognized on the native gel (Fig. 3). However, its presence in the leaves seemed to be evident from reproducible appearences on the gel assays.

POD Isozyme Profiles

CAT dismutates H_2O_2 into water and O_2, whereas peroxidase (POD) decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds (Campa et al., 1991). POD usually occurs as multiple isozymes in plants and has a number of potential roles in stress tolerance as well as in plant growth and development (Campa et al., 1991). Recently, plants are believed to be able to synthesize new isozymes with altered kinetic properties to alleviate oxidative damage under oxidative stress (Edward et al., 1994). Rao et al. (1996) have reported that one to four different POD isozymes could be distinguished in the leaves of *A. thaliana* according to their genotypes on the native gel. By their results, O_3 treatment as an inducer of oxidative stress enhanced the intensity of the pre-existing POD isozymes, but no new isozymes were synthesized in the leaves of *Arabidopsis* seedings. On the other hand, new synthesis of isozymes was found in flavonoid-deficient mutants irradiated with UV-B. Since AT can induce oxidative stress, we examined the changes in the POD isozyme profiles.

As shown in Figure 4, we observed three POD isozymes in *A. thaliana* leaves, but none of them were significantly affected by AT except for the loss of one weak isozyme (POD1) in AT-treated leaves at day 3. In fact, we obtained no significant changes in total POD activities with AT treatment (data not shown). Therefore, AT-induced CAT deactivation does not seem to affect on the POD isozyme profile in relation to the removal of H_2O_2 in *A. thaliana* leaves.

APX and GR Isozymes

Besides POD, APX (ascorbate peroxidase), and GR (glutathione reductase) are also suggested to act in conjunction in the removal of H_2O_2 through the metabolic cycle known as the ascorbate-glutathione cycle (Rao et al., 1996; Donahue et al., 1997). Although this cycle is known to be responsible primarily for H_2O_2 scavenging in chloroplasts, its importance in the cytosol is also becoming apparent (Klapheck et al., 1990; Donahue et al., 1997). In our previous study, we observed the enhanced total APX and GR activities to be concomitant with severe CAT deactivation in response to AT treatment in leaves of *A. thaliana* (Kang et al., 1998). Therefore, we speculated that this increase in APX and GR activities might be related to the activation of the ascorbate-glutathione cycle under the situation of CAT deactivation. The zymogram of *A. thaliana* leaves in this study revealed two APX isozymes; one (APX1) was prominent, and the other (APX2) was very faint (Fig. 5). The activity of major APX1 isozymes was strongly enhanced after 1 day of AT treatment; however, any new synthesis of APX isozymes was not observed. The fact that APX1 isozyme level was elevated in AT-treated leaves seems to indicate a certain role in stress tolerance. Similarly, Rao et al. (1996) reported that leaves of A. thaliana have two APX isozymes, and that ozone (O_3) exposure enhanced the intensity of isozyme activity. Oxidative stress is also known to modify the enzymic properties of GR in several plant species (Edward et al., 1994). Rao et al. (1995) reported a new GR isozyme synthesis due to O_3 exposure in leaves of wheat seeding plants. In the case of GR isozyme profiles, leaves of *A. thaliana* in this study had three isoymes in control plants (Fig. 6). The intensities of pre-existing two isozymes (GR2 and GR3) remained unaffected by AT treatment, whereas the intensity of

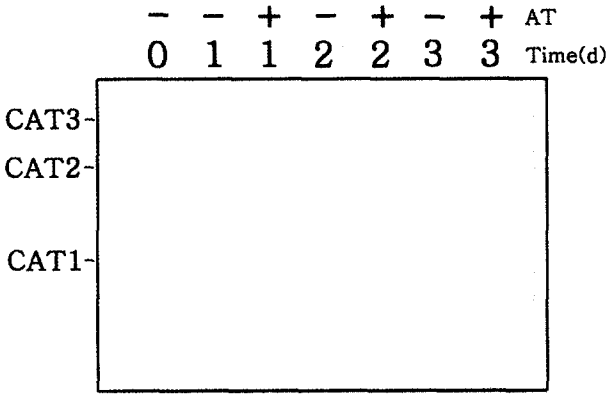

Figure 3. The native gel stained for the activity of CAT isozymes in leaves of *A. thaliana* grown in the presence (+) or absence (–) of 0.1 mM AT for 3 days. Equal amounts of protein (30 μg) from plants were loaded on the gel. The numbers on each lane indicate the time of AT treatment. The CAT1 isozyme activity was significantly inhibited from day 1 by AT treatment compared to control plants.

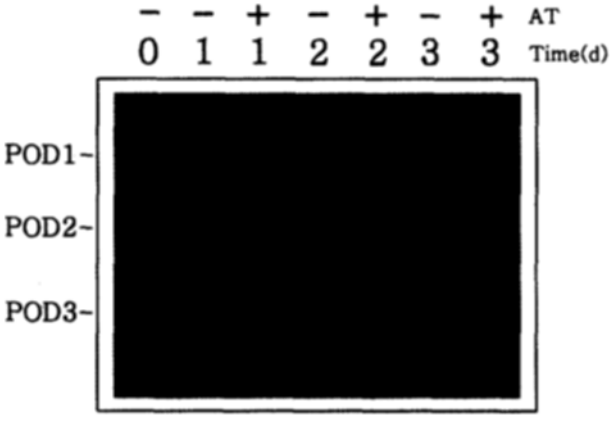

Figure 4. The native gel stained for the activity of POD isozymes in leaves of *A. thaliana* grown in the presence (+) or absence (–) of 0.1 mM AT for 3 days. Equal amounts of protein (120 μg) from plants were loaded on the gel. The numbers on each lane indicate the time of AT treatment.

GR1 isozyme was enhanced remarkably after 3 days of AT treatment. In particular, a new GR isozyme (the arrow, "←" in Fig. 6) was synthesized at day 3 in AT-treated leaves.

In conclusion, these results collectively mean that a striking inactivation of total CAT activity by AT in *A. thaliana* leaves resulted largely from the suppression of the CAT1 isozyme, and that APX1, GR1, and a newly synthesized GR isozyme could complement the role of CAT1 to metabolize H_2O_2 into non-toxic water.

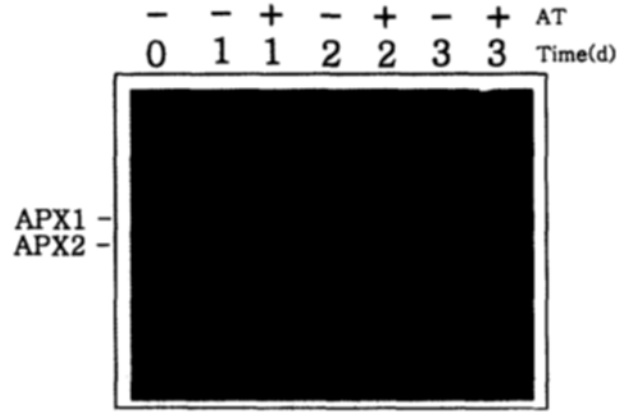

Figure 5. The native gel stained for the activity of APX isozymes in leaves of *A. thaliana* grown in the presence (+) or absence (–) of 0.1 mM AT for 3 days. Equal amounts of protein (120 μg) from plants were loaded on the gel. The numbers on each lane indicate the time of AT treatment. The intensity of APX1 was especially enhanced at day 3 after AT treatment.

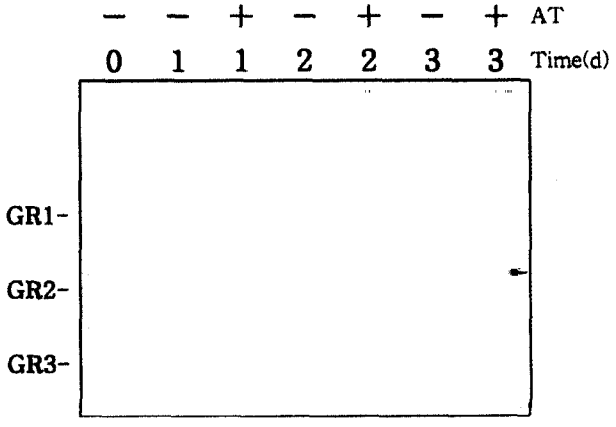

Figure 6. The native gel stained for the activity of GR isozymes in leaves of *A. thaliana* grown in the presence (+) or absence (–) of 0.1 mM AT for 3 days. Equal amounts of protein (100 μg) from plants were loaded on the gel. The numbers on each lane indicate the time of AT treatment. The staining intensity of the GR1 isozyme was significantly enhanced after 3 days of AT treatment compared to control plants. Also, a new GR isozyme (the arrow " ←") appeared at day 3 in AT-treated leaves, which was absent in control plants.

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