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Activation of Ascorbate-Glutathione Cycle in *Arabidopsis* Leaves in Response to Aminotriazole

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Aminotriazole(AT)-induced changes in growth, hydrogen peroxide content and activities of H_2O_2-scavenging antioxidant enzymes were investigated in the growing leaves of *Arabidopsis* plants (*Arabidopsis thaliana* cv Columbia). Catalase activity of rosette leaves was reduced by 65% with an application of 0.1 mM AT (a herbicide known as a catalase inhibitor), whereas the leaf growth and H_2O_2 content were almost unaffected. However, an approximate 1.6 to 2-fold increase in cytosolic ascorbate peroxidase (APX) activity concomitant with a substantial activation of glutathione reductase (GR) (approx. 22% increase) was observed during leaf growth in the presence of 0.1 mM AT. The activity of cytosolic APX in leaves was also increased by 1.8-fold with an application of exogenous 2 mM paraquat (an inducer of H_2O_2 production in plant cells) in the absence of AT. These results collectively suggest that (a) cytosolic APX and GR operate to activate an ascorbate-glutathione cycle for the removal of H_2O_2 under severe catalase deactivation, and (b) the expression of APX seems to be regulated by a change of the endogenous H_2O_2 level in leaf cells.

Keywords: *Arabidopsis thaliana*, aminotriazole, ascorbate-glutathione cycle, ascorbate peroxidase, glutathione reductase, H_2O_2.

When a plant is stressed, the production of activated oxygen species such as H_2O_2 and superoxides can exceed the capacity of the scavenging systems, resulting in oxidative damage (Price *et al.*, 1994; Anderson *et al.*, 1995). Thus, the ability of a plant to improve its activated oxygen scavenging capacity may be an important element for stress tolerance. The enzymic control of H_2O_2 at the cellular level is, therefore, a major feature of the protective mechanism in plant cells. Since H_2O_2 is a primary substrate for catalase and the removal of H_2O_2 is thought to be carried out exclusively by catalase, one might expect that a consequence of catalase deactivation could potentially allow H_2O_2 to increase to the toxic level. Plant catalase has been known to be localized largely in peroxisomes. On the other hand, H_2O_2 can easily permeate membranes and diffuses from peroxisomes to other cellular compartments (Scandalios *et al.*, 1972; Klapheck *et al.*, 1990). This leads us to speculate on the potentiality of catalase outside the peroxisomes and its relation to other enzymes involved in H_2O_2 scavenging metabolism. Scavenging of H_2O_2 by the ascorbate-glutathione pathway has been well demonstrated in plant chloroplasts (Foyer and Halliwell, 1976; Nakano and Asada, 1981; Foyer *et al.*, 1994). Ascorbate peroxidase(APX) catalyzes the first step of this H_2O_2 scavenging cycle by using ascorbate as a reductant (Foyer and Halliwell, 1976). Glutathione reductase (GR) is involved in the last step of this cycle by regenerating glutathione, which is the rate-limiting step of the cycle. Although this cycle is known to be responsible primarily for H_2O_2 scavenging in chloroplasts, its importance in the cytosol and in nonphotosynthetic tissues is also becoming apparent. For instance, Dalton *et al.* (1993) reported that legume root nodules use the ascorbate-glutathione cycle to remove toxic H_2O_2 for efficient nitrogen-fixation processes. In addition, this cycle has also been reported in endosperm cells of dark-germinating castor bean seeds which show metabolic events of extremely high turnover of H_2O_2 (Klapheck *et al.*, 1990). Recently, Ushimaru *et al.* (1997) showed that the activities of enzymes involved in this cycle are lower in rice seedlings

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which have been germinated under water than in aerobically grown controls. They also reported that these activities increase when the submerged seedlings are transferred to air. The above studies indicate the protective role of ascorbate-glutathione cycle in H_2O_2 removal in response to growth conditions and environmental stresses. However, there is little information concerning the relationship between catalase and ascorbate-glutathione cycle enzymes in relation to the control of H_2O_2 level within the same plant tissue cells under stressful conditions. So, we herein have followed the change of H_2O_2 level and developments of catalase and the ascorbate-glutathione cycle enzymes in *Arabidopsis* leaves treated with a herbicidal compound, aminotriazole. This herbicide is known to be a specific inhibitor of catalase and thus it has been used as a tool to artificially inhibit catalase activity and to initiate the accumulation of oxidative stress in plants (Prasad, 1997). In addition, we also examined the response of APX enzyme under treatment of paraquat (an inducer of H_2O_2 production in plant cells) to see a regulation of APX expression.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The surface-sterilized seeds (*Arabidopsis thaliana* cv Columbia) with 1% NaOCl for 10 min were sown and allowed to germinate in plastic pots (115×115×100 mm) containing commercial soils (Ball Seed Co., Canada) moistened with distilled water. After sowing, the pot was covered with transparent plastic film to prevent dessication for the first 4 days. *Arabidopsis* plants were grown in the growth chamber under a 18 h light/ 6 h dark regime, with 4,000 lux of light intensity, 70% RH, and day/night temperatures of 24/21°C. After 4 days of sowing, the plants were watered with half-strength Hoagland mineral solution on alternate days. Twelve-day-old plants were treated with the indicated concentrations of aminotriazole (AT) and grown further 5 days in the same growth chamber. Control plants were grown without AT treatment.

Measurement of Leaf Growth

Whole plants were sampled at daily intervals, and the fresh rosette leaves were rapidly weighed for determination of leaf growth.

Measurement of Chlorophyll and H_2O_2 Content

The chlorophyll extraction from the plant rosette leaves was done according to the method of Hiscox and Israelstam (1979). Five individual plant leaves were combined and weighed, and chlorophyll was extracted using DMSO solution. The chlorophyll concentration was measured as in Arnon (1949). For assay of H_2O_2 content, 15 plant rosette leaves were homogenized by mortar and pestle using 3 ml of 100 mM sodium phosphate buffer (pH 6.8). The homogenate was centrifuged at 18,000×g for 20 min and the supernatant collected for assay. Determination of H_2O_2 content was performed basically according to Bernt and Bergmeyer (1974) using a peroxidase enzyme. 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% (w/v) o-dianisidine, 40 μg peroxidase/mL) and then incubated at 30°C for 10 min in a waterbath. The reaction was stopped by adding a 0.5 mL of 1 N perchloric acid and centrifuged at 3,000×g for 3 min. The resultant clear supernatant was read at 436 nm and its absorbance was compared to the extinction of a H_2O_2 standard.

Preparation of Enzyme Extracts

For the study of activity development changes of antioxidant enzymes, ten plant rosette leaves were extracted with precooled mortar and pestle using 2 mL of 0.1 M K-phosphate, pH 7.5, 2 mM Na_2-EDTA and 1% PVPP. The supernatant, recovered at 4°C by centrifugation (24,000×g, 20 min), was used for enzyme assay. In the case of APX enzyme, 10 rosette leaves were homogenized in 2 mL of 0.1 M potassium-phosphate buffer (pH 7.5) containing 1 mM Na_2-EDTA, 1% PVPP and 5 mM ascorbate. The protein content in the extracts was determined according to Lowry *et al.* (1951) with BSA as a standard.

Enzyme Assay

Catalase activity was determined by monitoring the decrease of absorbance at 240 nm due to H_2O_2 (ε_{240}=39.4 $M^{-1}cm^{-1}$) consumption. The 3 mL reaction mixture contained 100 mM 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). Guaiacol peroxidase (GPX) activity was determined with guaiacol (ε_{470}=25.2

$mM^{-1}cm^{-1}$) at 470 nm following the method of Polle *et al.* (1994). The 3 mL reaction mixture contained 100 mM potassium phosphate, pH 6.5, 16 mM guaiacol, 10 mM H_2O_2 and enzyme extract. The reaction was initiated by adding enzyme extract. Ascorbate peroxidase (APX) activity was measured according to Asada (1984) from the decrease in absorbance at 290 nm as ascorbate was oxidized (ε_{290}=2.8 $mM^{-1}cm^{-1}$). The reaction mixture (2 mL) contained 50 mM potassium phosphate, pH 7.0, 0.5 mM ascorbate, 0.25 mM H_2O_2 and enzyme extract. Correction was done for the low, nonenzymatic oxidation of ascorbate by H_2O_2. Glutathione reductase (GR) activity was determined by following the oxidation of NADPH at 340 nm (ε_{340}=6.2 $mM^{-1}cm^{-1}$) as described by Rao *et al.* (1996). The reaction mixture (2 mL) contained 100 mM potassium phosphate, pH 7.8, 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG and enzyme extract. The assays were initiated by the addition of NADPH.

RESULTS AND DISCUSSION

Influence of Aminotriazole on Leaf Growth and Catalase

Aminotriazole is a herbicide that can be easily taken up into plant cells (Singer and McDaniel, 1982) and retards various plant metabolic processes such as pigment biosynthesis, chloroplast functions and root elongation growth (Heim and Larrinua, 1989). This herbicidal compound is also known as a specific inhibitor of catalase by directly binding to its protein moiety (Margoliash *et al.*, 1960). Therefore, aminotriazole has been used as a tool to artificially inhibit catalase activity and to initiate the accumulation of oxidative stress in plants (Prasad, 1997).

The present experiment was performed with determining the concentration of treatment of aminotriazole to *Arabidopsis* seedling plants. As shown in Fig. 1A, any significant effect of aminotriazole on the chlorophyll reduction (a major biochemical indicator on cell damages) in *Arabidopsis* leaves were not found up to 0.1 mM concentrations. However, the remarkable decrease in the chlorophyll content occurred at 1 mM aminotriazole. This indicates that the development of *Arabidopsis* leaves was not disturbed by a 0.1 mM concentration of aminotriazole. There were no differences in the developmental changes of fresh weight and the chlorophyll content in growing leaves over 5 days with or without 0.1 mM

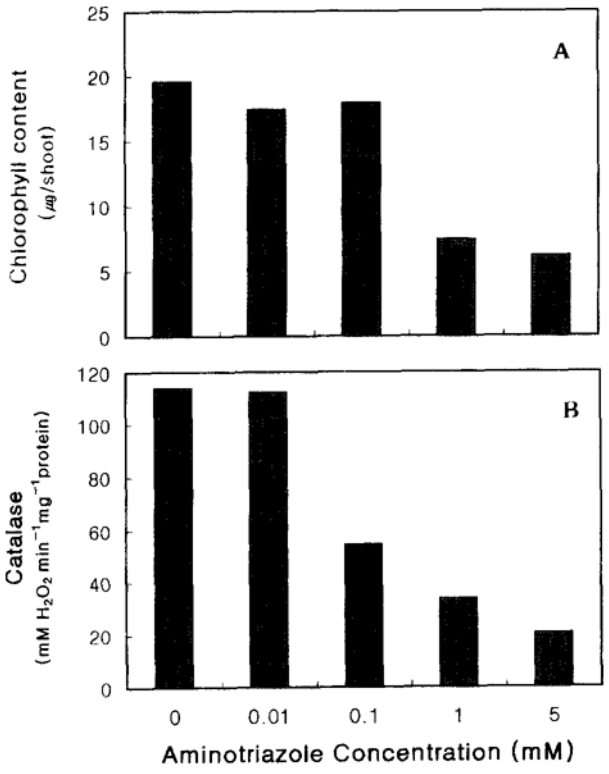

Fig. 1. Effects of concentrations of aminotriazole on the chlorophyll content (A) and the catalase activity (B) of the rosette leaves in *Arabidopsis thaliana* plants. Aminotriazole was treated when seedling plants were 12-day-old. Each values were determined after 5 day of treatment. The data presented are mean values of three independent replicate assays.

aminotriazole (Fig. 2). On the other hand, a 54% reduction in catalase activity was found at this 0.1 mM aminotriazole (Fig. 1B). Therefore, an aminotriazole concentration of 0.1mM was used in the subsequent experiments.

Developmental Changes of Catalase and Hydrogen Peroxide Level by Aminotriazole.

By using an 0.1 mM aminotriazole, we first tried to examine the relationship between catalase activity and H_2O_2 content in growing leaves. Since hydrogen peroxide is the primary substrate for catalase, one might expect that a consequence of catalase deactivation would be the accumulation of H_2O_2 and other toxic oxygen radicals, resulting in cell damages. Such changes account for degradative responses of plants to unfavorable environmental conditions including drought, high salinity and low temperature (Ferguson and Dunning, 1986; Price *et al.*, 1994).

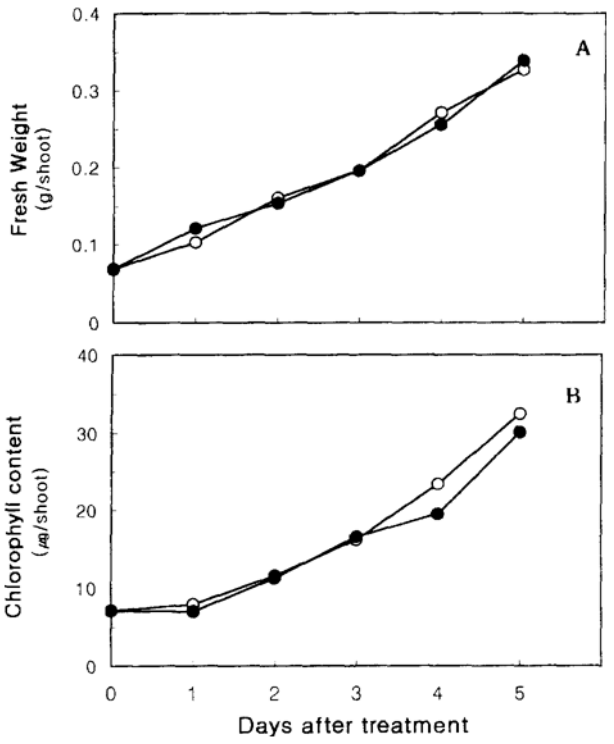

Fig. 2. The developmental changes of the fresh weight (A) and chlorophyll content (B) in growing leaves of *A. thaliana* plants over 5 days in the presence (●) or absence (○) of 0.1 mM aminotriazole. An aminotriazole treatment was initiated when seedling plants were 12-day-old. The data presented are mean values of three independent replicate assays.

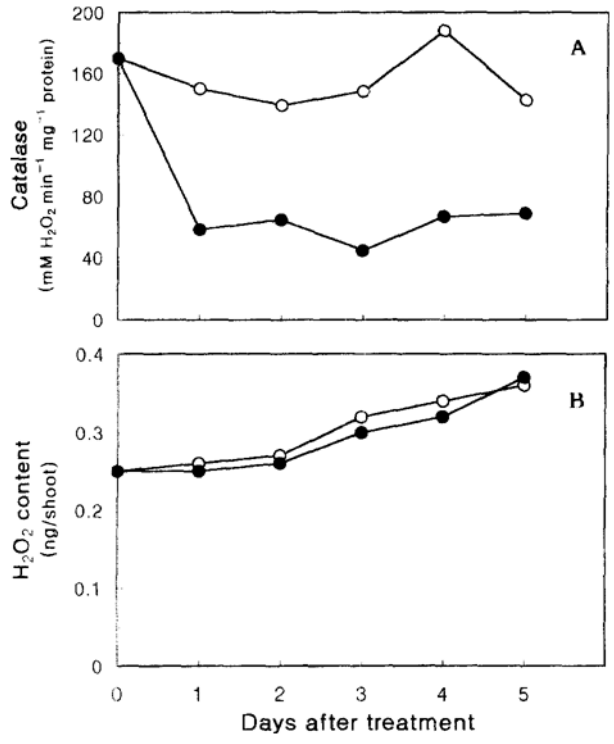

Fig. 3. The developmental changes of catalase activity (A) and hydrogen peroxide content (B) in growing leaves of *A. thaliana* over 5 days in the presence (●) or absence (○) of 0.1 mM aminotriazole. An aminotriazole treatment was the same as in Fig. 2. Each values are the means of three replicate experiments.

However, there were reports that H_2O_2 did not necessarily increase to toxic concentrations as a result of a reduction in catalase activity (MacRae and Ferguson, 1985; Ferguson and Dunning, 1986). To confirm this, we followed catalase activity and H_2O_2 content in *Arabidopsis* leaves with 0.1 mM aminotriazole application for 5 days (Fig. 3A and B). The catalase activity in control plants remained continuously high during the whole period, whereas an approximate 65% reduction in catalase activity in 0.1 mM aminotriazole-treated leaves was found after 1 day of treatment and then this deactivation continued thereafter (Fig. 3A). However, there was no significant difference in H_2O_2 content in both group of plants although the catalase activity in aminotriazole-treated plants was severely inhibited (Fig. 3B). These results indicate that an increase in H_2O_2 to toxic levels is not an automatic consequence of catalase inactivation. So, it was necessary to examine whether this low level of catalase was sufficient for the removal H_2O_2 produced in growing *Arabidopsis* leaves.

Activity Developments of Guaiacol Peroxidase (GPX), Cytosolic Ascorbate Peroxidase (APX) and Glutathione Reductase (GR)

In addition to catalase, various peroxidases such as GPX and APX are also reported to be the major H_2O_2-utilizing enzymes in plants (Puntarulo *et al.*, 1988; Asada, 1992). Ferguson and Dunning (1986) have suggested that the existence of a range of peroxidases in a number of cellular locations provides some flexibility to the cell in responding to increased peroxide production. Therefore, we tried to examine the roles of other peroxidases in the H_2O_2 removal in *Arabidopsis* leaves. Since GPX is well known as a general plant peroxidase which decomposes hydrogen peroxide by oxidation of phenolic compounds in the cell wall and cytosol (Asada, 1992; Rao *et al.*, 1996), we first investigated the time-course of its activity development (Fig. 4A)

Although GPX activities in both groups of plants

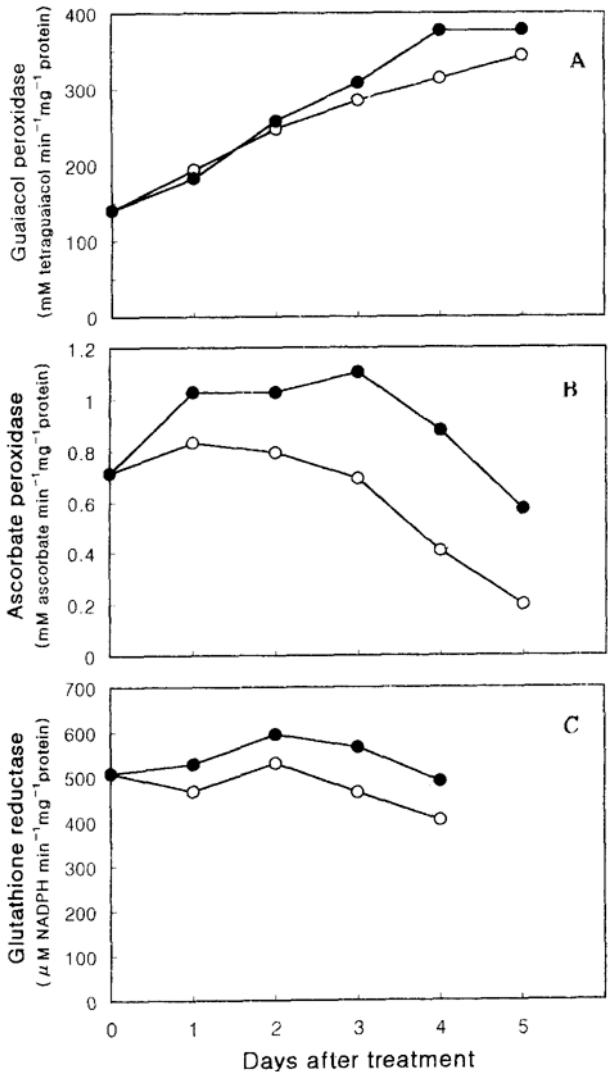

Fig. 4. Developmental changes of the activities of guaiacol peroxidase (A), cytosolic ascorbate peroxidase (B), and glutathione reductase (C) in growing leaves of *A. thaliana* over 5 days in the presence (●) or absence (○) of 0.1 mM aminotriazole. An aminotriazole treatment was the same as in Fig. 2. Each values are the means of three replicate experiments.

increased with the leaf age, any significant difference in the time course of activity development was not found (Fig. 4A). In fact, reduction in catalase activity by an aminotriazole treatment did not result in any elevated GPX activity.

It has been reported that APX is different from the typical plant peroxidases superfamily in terms of its structure and function, and it is also unique in having a preference toward ascorbate as a reductant (Chen and Asada, 1989; Mittler and Zilinskas, 1991; Asada, 1992). With the loss of catalase activity in aminotriazole- treated leaves, there is currently little detailed study on the metabolic role of cytosolic APX together with other antioxidant enzymes in H_2O_2 scavenging metabolism. Thus, we examined the development of activity of cytosolic APX in *Arabidopsis* leaves. Unlike GPX development, the cytosolic APX activity in aminotriazole-treated leaves was enhanced more rapidly for the first 3 days with an 1.6-fold increase compared to that of the control plants (Fig. 4B). Although APX activities in both groups of plants began to decline thereafter, an enhanced activity of APX by aminotriazole was consistently sustained. This result suggests a possibility that the cytosolic APX in *Arabidopsis* leaves might have a certain role in hydrogen peroxide decomposition under catalase deactivation due to an aminotriazole. Klapheck *et al.* (1990) have suggested that APX is as active as catalase in the degradation of H_2O_2 in dark germinating castor bean seeds. Moreover, the role of cytosolic APX toward oxidative stress conditions has been supported by recent works (Klapheck *et al.*, 1990; Dalton *et al.*, 1993; Ushimura *et al.*, 1997). These studies established a positive correlation between application of different oxidative stresses to an increase in APX activity.

It has been suggested that the ascorbate-glutathione cycle is an important constituent of the plant antioxidant system by playing a significant role in the H_2O_2 removal in the chloroplasts and cytosol of higher plants (Nakano and Asada, 1981; Rao *et al.*, 1995). APX and GR are two major enzymes that govern the cycle (Rao *et al.*, 1995). Although APX is the primary catalyzer of H_2O_2 to water, GR is also essential in this cycle in order to maintain the redox state of ascorbate and glutathione (Foyer *et al.*, 1994). The potential of APX to metabolize H_2O_2 depends on the redox state of such compounds. We investigated the development of GR in growing leaves treated with aminotriazole. Although there was no large increase in GR activity development by an 0.1 mM aminotriazole, an approx. 22% enhancement in activity was found substantially (Fig. 4C). These activations of cytosolic APX and GR collectively indicate that APX and GR in *Arabidopsis* leaves are believed to activate an ascorbate-glutathione cycle for the removal of H_2O_2 under severe catalase deactivation due to an aminotriazole.

Expression of Cytosolic APX in Response to Paraquat

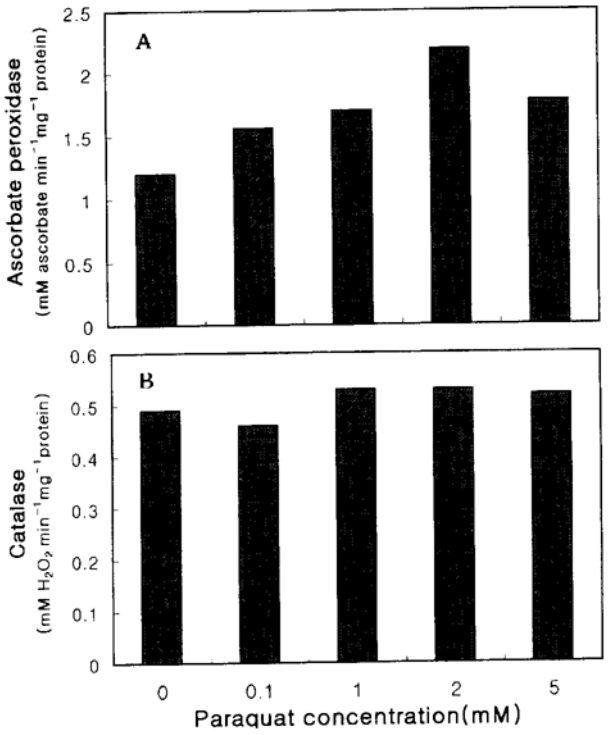

Fig. 5. Effects of paraquat on the activity developments of cytosolic ascorbate peroxidase (A) and catalase (B) of *A. thaliana* leaves. Paraquat was treated on 12-day-old seedlings. Ascorbate peroxidase activity was determined after 1 day of treatment and catalase activity was measured after 2 day of treatment. Each values are the means of three replicate experiments.

We observed an increase in cytosolic APX activity following a treatment with 0.1 mM aminotriazole in *Arabidopsis* leaves. In this case, the question arises as to how does an aminotriazole induce cytosolic APX activation? There is currently little information on the clear mechanism underlying its response to aminotriazole, although the uptake and metabolism of aminotriazole in plants have been studied (Feierabend and Schubert, 1978; Singer and McDaniel, 1982; Heim and Larrinua, 1989).

Klapheck *et al.* (1990) suggested that the increased endogenous H_2O_2 level in plant cells by external oxidative stress may cause an induction of APX activity. Although it seems unlikely, there was a report that highly reactive compounds like H_2O_2 or oxidative free radicals might function as secondary messengers in a narrow concentration range (Prasad *et al.*, 1994). We analyzed the effect of exogenously supplied paraquat (an inducer of H_2O_2 production in plant cells) on the expression of cytosolic APX in leaves in the absence of aminotriazole.

Figure 5(A) indicates that the cytosolic APX activity increased 1.8-fold by 2 mM paraquat compared to that of the control. However, the expression of catalase was almost unaffected (Fig. 5B). Therefore, this increase of APX activity by paraquat suggests that aminotriazole may cause an induction of APX through a modulation of leaf metabolic events related in H_2O_2 metabolisms in *Arabidopsis* plants.

In conclusion, cytosolic APX and GR enzymes are believed to activate an ascorbate-glutathione cycle in order to remove H_2O_2 under catalase deactivation due to an aminotriazole. In addition, the expression of APX appears to be controlled by an endogenous H_2O_2 level in leaf cells.

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