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Effects of abscisic acid on ozone tolerance of rice (*Oryza sativa* L.) seedlings

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Abstract

Ozone is one of the major gaseous pollutants detrimental to crop growth and metabolism. The objective of this research was to study how ABA ameliorates the effects of ozone on rice seedlings. Seedlings of two rice cultivars with different sensitivities to ozone (Tainung 67, tolerant; and Taichung Native 1, sensitive) were treated with 400 ppb of ozone or ABA and 400 ppb of ozone to determine their effect on growth, stomatal movement, chlorophyll characteristics, and the activity of antioxidant enzymes. Activities of the enzymes SOD, APOD, GR and POD were significantly higher in the sensitive cultivar, TN 1, than in the tolerant cultivar, TNG 67. Seedlings of the sensitive cultivar pretreated with ABA (10 μM) were significantly more tolerant of ozone than control seedlings. Pretreatment with ABA effectively reduced stomatal conductance and the degree of injury. Abscisic acid also increased ascorbate peroxidase and glutathione reductase activity. Ozone increased peroxidase activity in sensitive seedlings, but ABA decreased peroxidase activity. The sensitive cultivar had a higher density of stomata on its leaves than the tolerant cultivar. The results suggest that ABA induced tolerance to ozone may be more associated with its effects on stomatal movement than on the modulation of antioxidant enzyme activity.

Introduction

Ozone is one of the major air pollutants causing physiological damage to both animals and plants. Ozone can significantly reduce crop yield (Heck et al. 1983). Long term exposure of rice to 10–20 nl L^{-1} ozone resulted in grain yield losses up to 40% (Kats et al. 1985; Kobayashi et al. 1995; Wahid et al. 1995). Understanding the physiological responses to ozone and the mechanisms that confer tolerance to ozone should help prevent crop yield reduction.

In plants, many physiological processes can be affected by exposure to ozone. Ozone can reduce quantum yield, electron transport of photosystem II, membrane permeability, stomatal conductance, the function of enzymes for CO_2 fixation and, ultimately, photosynthesis (Darrall 1989; Kangasjärvi et al. 1994). Initially, the damage may be caused by active oxygen species and hydrogen peroxide released by ozone attack on apoplastic structures (Kangasjärvi et al. 1994). However, plants may ameliorate ozone injury through stomate closure and induction of the Halliwell-Ashada pathway for scavenging active oxygen species and hydrogen peroxide (Nouchi 1993; Pell et al. 1997). Some plant hormones, such as ethylene, polyamines and abscisic acid (ABA), are also involved in the regulation of plant defenses against ozone (Adepipe and Ormrod 1972; Downton et al. 1988; Jeong et al. 1980; Jeong and Ota 1981).

In a previous study, we found that japonica type rice cultivars were more tolerant of ozone than indica type cultivars (Yang et al. 2000). Exposure to 300 ppb of ozone for 4 h can cause significant, visible damage to the leaf tips of the sensitive, Taichung Native 1 (TN 1) indica type cultivar. However, the tolerant, japonica type cultivar Tainung 67 (TNG 67), did not suffer visible damage until exposed to 800 ppb ozone for 4 h. Preliminary results from the sensitive cultivar, TN 1, showed that ozone could cause decreases in chlorophyll and soluble leaf proteins, increases in membrane leakage and malondialdehyde (MDA), and differential regulation of enzymes in the Halliwell-Ashada pathway. In addition, application of ABA could significantly reduce damage caused by ozone fumigation (Yang et al. 2000). Thus, the objective of this study was to determine the physiological functions of ABA in ameliorating ozone injury and the mechanisms conferring cultivar tolerance to ozone.

Materials and methods

Plant material

Rice (*Oryza sativa L.*) seedlings were cultured as described in Lee et al. (1993). Rice seeds (cv. TN 1, ozone sensitive, and cv. TNG 67, ozone tolerant) were sterilised with 5% NaOCI, and germinated at 37 °C. Germinated seeds were cultured with half-strength Kimura Solution in a phytotron at 30/25 °C (day/night) and RH between 65–85%, under natural photon flux density.

Pretreatment with ABA

To evaluate the effect of ABA on the amelioration of ozone damage, seedlings at the three-leaf stage were treated for 24 h with 10 μM ABA from a culture solution. Seedlings were then fumigated with ozone and used for physiological analyses.

Ozone fumigation

Ozone fumigation was performed in a chamber with continuously circulated charcoal-filtered air. Temperature was maintained at 25–30 °C, and RH was kept at 70 ± 5%. Photo flux density was around 340 μmole m^{-2} s^{-1}. Ozone was continuously generated from pure O_2 using an ozonizer. Ozone concentration in the chamber was monitored by a detector (Thermo Environment 49, Thermo Environmental Instruments Inc., Franklin, MA, USA). Seedlings were exposed to 400 ppb of ozone for 4 h, and then moved to the phytotron for recovery.

Damage assessment

The severity of injury was visually determined using the scale and descriptions developed by the International Rice Research Institute (International Rice Research Institute 1996). The levels of injury, which were based on the extent of brown spots on the leaf tips of seedlings, were 1 (no damage), 3, 5, 7, and 9 (dead).

Leaf stoma density and stomatal conductance

The 3rd leaf of each seedling was used for this analysis. The area of each leaf was measured with an area meter (LI-3000A, LI-COR Inc., Neb, USA). Each leaf was also smeared with a film of fingernail polish. After the polish dried, the film print was used to count stomates with a microscope. Stomate density was expressed as the number of stomates per mm^{-2} leaf area.

After seedlings were pretreated with ABA for 24 h and exposed to ozone for 4 h, leaf stomatal conductance was determined using the method described in our previous report (Lee et al. 1993).

Chlorophyll and soluble protein

Our preliminary results showed that the symptoms of ozone injury appeared mostly in the apical region of leaves. Thus, the apical 3 cm of the 3rd leaf of each seedling was cut off and used for chemical content and enzyme activity analyses. Chlorophyll content was determined as described by Wintermans and De Mots (1965), the methods of Chang and Kao (1998) were used for analysis of soluble proteins in leaves, and protein content was quantified using the methods in Bradford (1976).

Peroxide and malondialdehyde

Leaf peroxide was extracted with sodium phosphate buffer (50 nm pH 6.8) and estimated according to Jana and Choudhuri (1982). Malondialdehyde was extracted with 5% trichloroacetic acid and quantified as described in Heath and Packer (1968).

Chlorophyll fluorescence

Fluorescence analysis was conducted as described in Guidi et al. (1997). Chlorophyll fluorescence was determined by a chlorophyll fluorometer (Heinz Walz. PAM-200, Effeltrich, Germany). The activity of PSII was expressed as the ratio of maximal fluorescence (Fm) to variable fluorescence (Fv), Fm/Fv.

Enzyme analysis

The apical 3 cm of the 3rd leaf of seedlings were dissected, homogenised with sodium phosphate buffer (50 nm, pH 6.8), and centrifuged at 12,000 g, 4 °C for 20 min. Aliquots of the supernatants were used for enzyme assays.

Superoxide dismutase (SOD) activity was assayed as described by Paoletti et al. (1986). Peroxidase activity (POX) was determined by the method of Curtis and Howell (1971) and Kato and Shimizu (1987). Catalase (CAT) activity was measured according to Kato and Shimizu (1987) And the activity of ascorbate peroxidase (APOD) was determined as described in Nakano and Asada (1981). Results were based on at least 4 replicates of each enzyme activity assay.

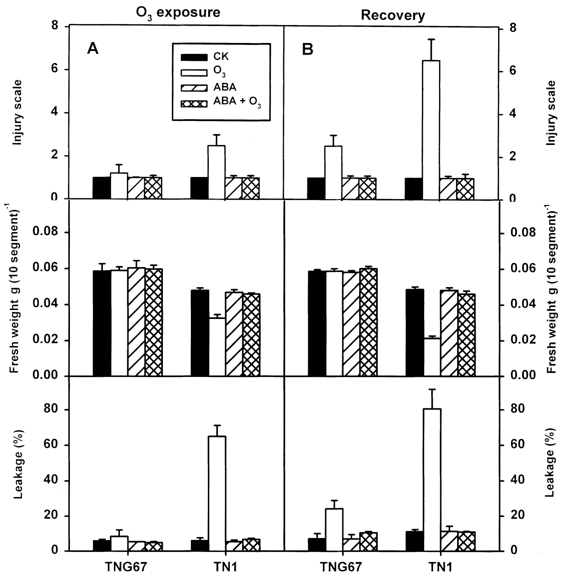

Figure 1. Degree of injury, fresh weight, and electrolyte leakage of the leaves of rice seedlings immediately after exposure to 400 ppb O_3 for 4 h (left column) and after 24 h of recovery (right column). Bars indicate standard errors (n = 4).

Results

Injury scale, chlorophyll, and protein content

Cultivar TN 1 suffered significant injury from 4 h of ozone exposure, with some seedlings classified as grade 6 or more after 24 h of recovery (Figure 1, upper panel). Leaves of the tolerant cultivar, TNG 67, were only slightly injured. Treatment with ABA 24 h before ozone fumigation effectively prevented ozone damage to both TN 1 and TNG 67.

Ozone exposure significantly reduced the fresh weight of the sensitive cultivar (Figure 1, middle panel), which is consistent with the extent of its injuries. TN 1 apparently experienced higher electrolyte leakage than TNG 67 (Figure 1, bottom panel), suggesting that its membranes were damaged by the ozone treatment. Pretreatment with ABA significantly reduced the effect of ozone on fresh weight and electrolyte leakage.

Ozone reduced chlorophyll content less than soluble protein content (Figure 2). In TN 1, chlorophyll content was only slightly decreased by ozone, but even after 24 h of recovery protein content was only 1/3 that of control (Figure 2, bottom panel, right side).

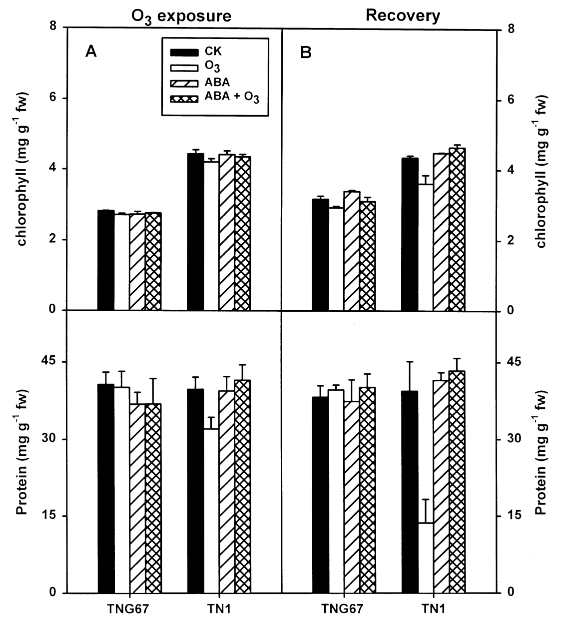

Figure 2. Chlorophyll and soluble protein content in the leaves of rice seedlings immediately after exposure to 400 ppb O_3 for 4 h (left column) and after 24 h of recovery (right column). Bars indicate standard errors (n = 4).

Malondialdehyde and chlorophyll fluorescence

In TN 1 seedlings, ozone treatment significantly elevated leaf MDA content, and reduced the Fv/Fm ratio remarkably (Figure 3). The increase in leaf MDA content reflected a higher lipid peroxidation status in ozone treated seedlings, and the lower Fv/Fm ratio indicated that ozone decreased PSII electron transfer efficiency. The leaf MDA content and Fv/Fm ratio of TN 1 seedlings treated with ABA and then exposed to ozone were similar to those of control seedlings.

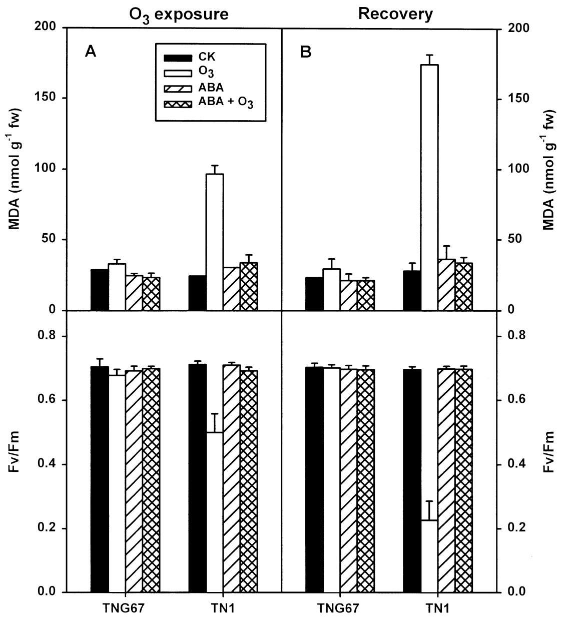

Figure 3. The Fv/Fm ratio and MDA level in the leaves of rice seedlings immediately after exposure to 400 ppb O_3 for 4 h (left column) and after 24 h of recovery (right column). Bars indicate standard errors (n = 4).

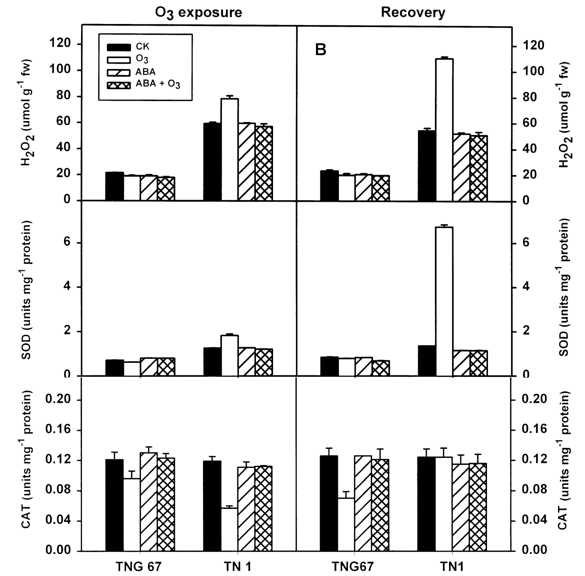

Figure 4. H_2O_2 levels, and SOD and CAT activity in the leaves of rice seedlings immediately after exposure to 400 ppb O_3, for 4 h (left column) and after 24 h of recovery (right column). Bars indicate standard errors (n = 4).

Thus, application of ABA significantly ameliorated the effect of ozone on both physiological processes.

Superoxide and enzyme activity

In general, seedlings of the sensitive cultivar, TN 1, had a higher H_2O_2 content than seedlings of the tolerant cultivar, TNG 67 (Figure 4). Exposure to ozone significantly increased H_2O_2 content in TN 1, and this increase continued during recovery stage. Pretreatment with abscisic acid effectively reduced the internal H_2O_2 content during and after ozone exposure.

Following ozone exposure, SOD activity in TN 1 was higher than in TNG 67, especially during the recovery stage. The significant increase in SOD activity was associated with the high concentration of H_2O_2 in TN 1. SOD activity in TN 1 seedlings pretreated with abscisic acid was similar to that in control seedlings. During ozone exposure, catalase activity was significantly lower in both TNG 67 and TN 1 seedlings than in control seedlings (Figure 4, bottom panel). Pretreatment with ABA significantly enhanced catalase activity during the ozone treatment for both cultivars.

In general, ozone fumigation increased the activity of POD, APOD and GR in TN 1, and this increased enzyme activities continued during recovery stage

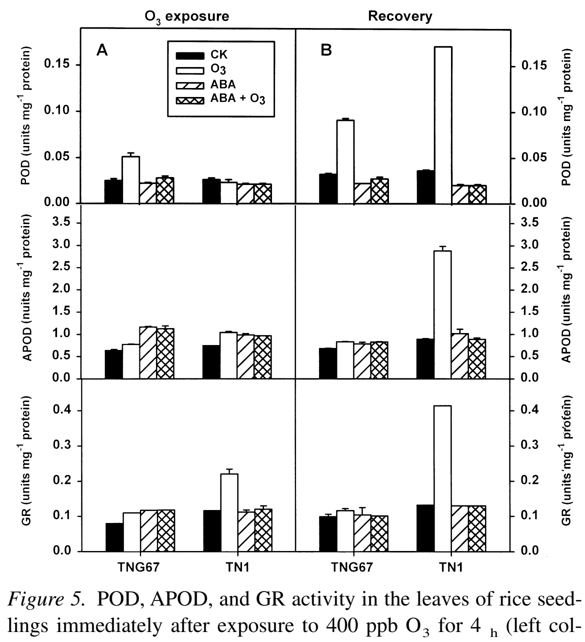

Figure 5. POD, APOD, and GR activity in the leaves of rice seedlings immediately after exposure to 400 ppb O_3 for 4 h (left column) and after 24 h of recovery (right column). Bars indicate standard errors (n = 4).

(Figure 5). In both cultivars, abscisic acid only slightly increased APOD activity during ozone exposure (left side of APOD panel).

Table 1. Stomatal density and total leaf area of rice seedling.

Cultivar	Stomatal density (no./mm^2)	Total leaf area (cm $_2$)
TNG67	42.93	165.93
TN1	65.40[a]	335.78[a]

[a]Significant at the 0.01 probability level between TNG67 and TN 1 by t-test.

Table 2. Effects of ABA and 400 ppb ozone treatments on stomata] conductance of rice seedlings. In ABA treatment, seedlings were pretreated with ABA (10 μM) for 24 h before ozone fumigation for 4h.

Cultivar	Treatment			
	Control	O_3	ABA	ABA + O_3
	Stomatal conductance (μ mol H_2O cm^{-2} s^{-1})			
TNG67	136.3 Aa[a]	71 .74 Ab	39.93 Ac	43.12 Ac
TN I	1 52.6 Ba	63.98 Bb	39.94 Ac	39.40 Ac

[a]Values followed by different lower case letters refer to the significance at the 0.01 probability level between treatments by LSD test. Values followed by different capital letters refer to the significant at the 0.01 probability level between TNG67 and TN 1 by t-test.

Stomatal density and conductance

The sensitive cultivar, TN 1, had more stomata per unit of leaf area and larger leaves than the tolerant cultivar, TNG 67 (Table 1). TN 1 also had higher stomatal conductance before ozone fumigation than TNG 67 (Table 2). In both cultivars, 4 h of ozone exposure reduced stomatal conductance to similar levels. For TN 1 and TNG 67, pretreatment with ABA effectively decreased stomatal conductance.

Discussion

It has long been known that different varieties of rice differ in their response to ozone, although the physiological basis for ozone sensitivity or tolerance was largely unknown (Jeong et al. 1980). A sensitive (TN 1) and a tolerant (TNG 67) cultivar of rice were selected from 1 2 japonica/indica type cultivars exposed to ozone (Yang et al. 2000). Ozone caused significant, visible damage to TN 1 seedlings, significantly increased membrane leakage, and MDA and H_2O_2 levels, and significantly decreased the Fv/Fm ratio, and the chlorophyll and soluble protein content of seedling leaves. These responses were consistent with those of other crops exposed to ozone (Guidi et al. 1997; Kangasjärvi et al. 1994). Increases in MDA and H_2O_2 following ozone treatment suggest that the high membrane electrolyte leakage may result from membrane lipid peroxidation and oxidative bursting of cell membranes (Chang and Kao 1998). Reduction in the Fv/Fm ratio, and, leaf chlorophyll and soluble protein content may decrease the rate of photosynthesis in TN 1 seedlings exposed to ozone.

Pretreatment with ABA for 24 h prevented ozone damage in TN 1. In TN 1, pretreatment with ABA seemed to ‘normalize’ all the physiological traits we evaluated under ozone fumigation, and made them similar to those of TNG 67. These results support the suggestion that ABA is involved in the physiological processes that confer ozone tolerance to rice cultivars (Jeong et al. 1980).

Enzymes for scavenging deleterious, active oxygen species can be induced by ozone in several crops, including rice (Nouchi 1993). It has been suggested that the elevated activity of certain enzymes in response to ozone is part of a protective system (Kangasjärvi et al. 1994). In our case, however, activities of the enzymes SOD, APOD, GR and POD were significantly higher in the sensitive cultivar, TN 1, than in the tolerant cultivar, TNG 67, suggesting that other physiological processes may confer ozone tolerance to ozone tolerance to TNG 67 and ABA treated TN 1.

Most ozone enters plants through the stomata. The density and opening of leaf stomata may also determine the sensitivity of plants to ozone (McLaughlin and Taylor 1981; Rich et al. 1970). TN 1 seedlings had significantly larger leaves and more stomata per unit leaf area than TNG 67 seedlings (Table 1), which may lead to higher leaf conductance and greater uptake of ozone into TN 1 leaves. Once ozone enters the stomata, it reacts instantaneously with apoplastic cell structures and generates active oxygen species and hydrogen peroxide (Kangasjärvi et al. 1994). Thus, TN 1 seedlings accumulated higher concentrations of MDA and H_2O_2 in ozone fumigated leaves. Although the stomata of TN 1 could be closed to some extent (Table 2) and the system for scavenging active oxygen species was induced by 4 h of ozone exposure, these physiological processes may not be fast enough to ameliorate the damage that is caused as ozone enters the leaf. The slow response of TN 1 stomata to chilling stress has been detected in our lab (Lee et al. 1993). On the other hand, pretreatment of TN 1 with ABA significantly facilitated stomatal closure and prevented the ingress of ozone (Table 2). Thus, reducing stomatal conductance may be a major factor conferring acclimation to ozone to TN 1, although ABA had some effect on the activity of several enzymes. Whether ABA also regulates ozone detoxification processes, such as adjustment of apoplastic pH or the reaction between ozone and ascorbate, still needs to be studied (Plöchi et al. 2000). In addition, experiments using more genotypes or cultivars are needed to generalize the realtionships among stomatal response, ABA, and ozone tolerance in rice.

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