Effects of Ozone on Photosynthetic Components and Radical Scavenging System in Leaves of Rice (*Oryza sativa* L.)

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Abstract

The chronic effects of ambient levels of O₃ on photosynthetic components and radical scavenging system of leaves at different positions on the main stem of Japanese rice (*Oryza sativa* L.) were investigated. Two cultivars of Japanese rice (Koshihikari and Kinuhikari) were planted in plastic boxes filled with flooded Andisol in 9 chambers on 15 May, and exposed daily to charcoal-filtered air or O₃ at 60 or 100 nl l⁻¹ (ppb) (10:00–17:00) from 30 May to 20 September 2007.

The activity and concentration of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) in the leaves of both cultivars were not significantly affected by exposure to O₃. However, the concentrations of chlorophyll in the lower leaves on the main stem of both cultivars were significantly reduced by the exposure to 100 ppb O₃, while the exposure to O₃ significantly increased the activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR); regardless of the leaf position in the two cultivars. Although no significant combined effect of O₃ and cultivar on the concentration of chlorophyll, the activity and concentration of Rubisco, or the concentration of antioxidants and activity of radical scavenging enzymes was observed, there was a highly positive correlation between the AOT40 (accumulated exposure over a threshold of 40 ppb) of O₃ and the relative activity of APX, MDAR or GR in leaves exposed to O₃ to that in leaves exposed to charcoal-filtered air of both cultivars. These results suggest that APX, MDAR and GR played important roles in the antioxidative response of Japanese rice to O₃. However, the detoxification capacity of reactive oxygen species by APX, MDAR and GR was insufficient to prevent the adverse effects of ambient levels of O₃ on the concentration of chlorophyll in the two cultivars of Japanese rice.

Key words: Antioxidant, *Oryza sativa*, Ozone, Radical scavenging enzymes, Rubisco.

1. Introduction

Tropospheric ozone (O₃) is a major component of photochemical oxidants and generated by photochemical reaction in the atmosphere under sunlight (Chameides *et al.* 1994). Recent modeling work by the United Kingdom Meteorological Office, using the IPCC-A2 emission scenario, indicates possible expansion of the area subjected to elevated summertime O₃ in coming decades with hotspots in North and South America, Eu-

rope and Asia (Collins *et al.* 2000). Recently, relatively high concentrations of O₃ have been observed in East Asia (Zheng and Shimizu 2003). In Japan, according to the ambient air quality data of the Atmospheric Environmental Regional Observation System in the National Institute for Environmental Studies, relatively high concentrations of O₃ above 100 nl l⁻¹ (ppb) have frequently been detected in suburban areas and the average concentration of O₃ has been rising year by year (Ohara *et al.* 2007). Relatively high concentrations of O₃ induce visible foliar injuries, inhibition of net photosynthesis and a reduction in growth and yield

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of many crops (Fuhrer *et al.* 1997; Heck *et al.* 1982; Nouchi *et al.* 1991). Furthermore, it has been reported that ambient levels of O_3 in suburban areas of Japan might induce a reduction in the growth and yield of several crops (Kobayashi 1988).

Ozone enters the leaves of crops primarily through the stomata. Interacting with water in the leaf extracellular spaces, O_3 degrades into reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and other free radicals (Hoigné and Bader 1976). Ozone-induced ROS may damage biomolecules in leaf cells such as membrane lipids, proteins and nucleic acids (Hippeli and Elstner 1996; Fuhrer and Booker 2003). Furthermore, several researchers reported that O_3 -induced ROS in the leaves of crops caused a reduction in the amount of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) and the inhibition of photosynthetic activity (Dann and Pell 1989; Farage *et al.* 1991).

The difference in the sensitivity to O_3 among the cultivars of a crop might be related to the detoxification capacity for O_3 -induced oxidative damage in the leaves (Cheng *et al.* 2007; Izuta *et al.* 1999; Fuhrer and Booker 2003; Nouchi 1993). For example, Cheng *et al.* (2007) reported that the detoxification capacity of O_3 -tolerant cultivar exceeded that of the O_3 -sensitive cultivar in soybean. In many crops, O_3 -induced ROS can stimulate the synthesis of antioxidative metabolites and enhance the activity of radical scavenging enzymes. For example, O_2^- is eliminated by superoxide dismutase (SOD) in a reaction that yields hydrogen peroxide (H_2O_2). Hydrogen peroxide (H_2O_2) is then scavenged by ascorbate peroxidase (APX) and catalase (CAT), whereupon ascorbate is regenerated by the reduction of monodehydroascorbate radical by monodehydroascorbate reductase (MDAR) and NADPH. The glutathione pathway is catalyzed by dehydroascorbate reductase (DHAR) and glutathione reductase (GR). It has been reported that the levels of certain antioxidants and the activity of several radical scavenging enzymes in rice were increased by exposure to O_3 (Nouchi 1993; Yoshida *et al.* 1994a; Yoshida *et al.* 1994b). However, the detoxification capacity depends on the concentration of O_3 , exposure period, age of plant tissues and genotype (Cheng *et al.* 2007; Bender *et al.* 1994).

Rice (*Oryza sativa* L.) is a primary agricultural crop in Japan. The short-term effect of O_3 on the photosynthetic components and radical scavenging systems of Japanese rice leaves were reported (Nouchi

et al. 1991; Nouchi 1993; Yoshida *et al.* 1994a; Yoshida *et al.* 1994b). However, no information concerning the long-term (chronic) effects of ambient levels of O_3 on photosynthetic components and radical scavenging systems of Japanese rice leaves is available. In the present study, we investigated the chronic effects of ambient levels of O_3 on the concentration of chlorophyll, activity and concentration of Rubisco, concentration of antioxidants and the activity of radical scavenging enzymes in the leaves at different positions on the main stem of two cultivars of Japanese rice.

2. Materials and Methods

2.1 Plant materials and gas treatment

In the present study, two cultivars of Japanese rice, Koshihikari and Kinuhikari, were used as plant materials. These are early to medium maturing cultivars (National Institute of Crop Science Rice Breeding Research Team, 2007). Rice seeds were obtained from the Field Production Science Center in the University of Tokyo. The 1500 seeds pre-germinated in water for 3 days were sown into seedling boxes (60 cm $\times$ 33 cm and a depth of 30 mm) filled with cultivation molding on 20 April, 2007. After incubation in a greenhouse, the seedlings were planted in plastic boxes (42.5 l) filled with flooded andisol (32.4 l) on 15 May, and grown until 20 September, 2007 in 9 chambers located at the Field Museum Tamakyuryo of Tokyo University of Agriculture and Technology (Hachioji, Tokyo). Fertilizer (N-P-K=14:14:14) was applied before planting at 25 g per box. Each chamber was 2.2 m width $\times$ 2.6 m depth $\times$ 2.0 m height. For each cultivar, 6 plants were planted in a plastic box, and 12 boxes were assigned to each chamber for a total of 648 plants.

The two cultivars were exposed daily to charcoal-filtered air or O_3 at 60 nl l⁻¹ (ppb) or 100 ppb (10:00-17:00) with 3 chamber replications from 30 May to 20 September 2007. The whole-plant dry mass and leaf area per plant at the beginning of the exposure to O_3 (29 May, 2007) were 141.1 $\pm$ 5.9 mg and 21.2 $\pm$ 1.7 cm² for Koshihikari, and 135.9 $\pm$ 1.5 mg and 15.4 $\pm$ 0.7 cm² for Kinuhikari, respectively. Ozone was generated from oxygen-enriched dry air via an electrical discharge O_3 generator (OZC-05A, Dylec Inc., Japan). The concentration of O_3 in each chamber was independently and continuously monitored at 30-minute intervals with a UV absorption O_3 analyzer (MODEL 1210, Dylec Inc., Japan), while the average concentration

and AOT40 of O₃ from 30 May to 20 September 2007 were calculated based on the monitoring data of O₃ concentration in the chambers and the AOT40 was calculated as the sum of differences between the hourly mean O₃ concentration and 40 ppb when the hourly mean O₃ concentration exceeded 40 ppb. The daylight AOT40 is the AOT40 during daylight hours when the solar radiation exceeded 50 W m⁻². The average 7-hour O₃ concentration between 10:00 and 17:00 and daylight AOT40 of O₃ from 30 May to 20 September 2007 were 20 ppb and 0.4 μl l⁻¹ h (ppm h) in the CF treatment, 58 ppb and 15 ppm h in the 60 ppb O₃ treatment, and 93 ppb and 42 ppm h in the 100 ppb O₃ treatment, respectively. The daily mean, maximum and minimum air temperatures in the chambers during the exposure period of rice from 30 May to 20 September, 2007 were 23.8°C, 30.9°C and 19.7°C, respectively.

2.2 Harvest of rice leaves for biochemical analysis

For biochemical analysis, leaves in different positions on the main stem, which were grown for approximately one month from the leaf emergence, were used. In the leaves of Koshihikari, the harvest date and the days from full expansion were 28 June and 21 days in the 8th leaf, 17 July and 29 days in the 10th leaf, 31 July and 28 days in the 12th leaf, and 21 August and 15 days in the 15th leaf, respectively. The 15th leaf of Koshihikari was the flag leaf. In the leaves of Kinuhikari, the harvest date and the days from full expansion were 29 June and 22 days for the 7th leaf, 18 July and 30 days for the 9th leaf, 1 August and 29 days for the 11th leaf, and 22 August and 16 days for the 14th leaf, respectively. The 14th leaf of Kinuhikari was the flag leaf. The leaves of both cultivars were harvested prior to the start of daily exposure to O₃ (5:00-9:30). After the leaf area and fresh weight had been measured, the leaves were frozen in liquid nitrogen as soon as possible and then stored at -80°C until use.

2.3 Measurement of the concentrations of chlorophyll and total soluble protein

To measure the concentrations of chlorophyll and total soluble protein (TSP), and the activity and concentration of Rubisco, the leaf samples (100 mg FW) stored at -80°C were homogenized to a fine powder in liquid nitrogen with a small amount of quartzose sand. Subsequently, the leaf samples were added to an extraction buffer of 1 ml containing 100

mM HEPES-KOH (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF and 20 mg of PVPP and the crude homogenate was centrifuged at 16,000 g for 10 minutes at 4°C. The supernatant of the sample was used in the assay of the concentration of TSP and the activity and concentration of Rubisco.

The concentration of chlorophyll was determined by the method of Barnes *et al.* (1992). After 150 μl of the crude homogenate was added to 1,350 μl of dimethyl sulfoxide in micro tubes, the samples were heated for one hour at 60°C and the absorbency of chlorophyll extracted from the leaf samples was measured at 664.9 and 648.2 nm with a spectrophotometer (UV-1240, Shimadzu, Japan). The concentration of chlorophyll was determined by the following formulas:

$$\begin{aligned} \text{Chlorophyll } a \text{ } (\mu\text{g ml}^{-1}) \\ = (14.85 \times A_{664.9}) - (5.14 \times A_{648.2}) \end{aligned}$$

$$\begin{aligned} \text{Chlorophyll } b \text{ } (\mu\text{g ml}^{-1}) \\ = (25.48 \times A_{648.2}) - (7.36 \times A_{664.9}) \end{aligned}$$

$$\begin{aligned} \text{Chlorophyll } a + b \text{ } (\mu\text{g ml}^{-1}) \\ = (7.49 \times A_{664.9}) - (20.34 \times A_{648.2}) \end{aligned}$$

The concentration of TSP was determined by the Bradford dye-binding procedure (Bradford 1976). To measure TSP, 30 μl of supernatant of the sample was added to 4.5 ml of dye solution containing 0.01% (w/v) CBB G-250, 5% (v/v) ethanol and 8.5% (v/v) phosphoric acid. The absorbency of the sample was measured at 595 nm within 5-30 minutes using a spectrophotometer. In the quantification of TSP, a calibration curve of the protein concentration was made with bovine serum albumin (BSA).

2.4 Measurement of the activity and concentration of Rubisco

The activity of Rubisco was determined by the modified method of Tissue *et al.* (1993). To measure Rubisco activity, the supernatant was applied to the PD-10 desalting column filled with Sephadex G-25 to desalt the supernatant, while to determine the initial activity of Rubisco, immediately after combining the desalted sample solution (150 μl) with an assay solution (830 μl) containing 50 mM HEPES-KOH (pH 8.0), 10 mM NaHCO₃, 0.2 mM NADH, 2.5 mM ATP, 10 mM KCl, 1 mM EDTA, 20 mM MgCl₂, 5 mM DTT, 5 mM phosphocreatine, 6 units ml⁻¹ phosphoglyceric kinase, 6 units ml⁻¹ glyceraldehyde 3-phosphate dehydrogenase and 20 units ml⁻¹ phosphocreatine kinase at the final

concentration, the reaction was started by adding 20 μ l of 30 mM RubP. The change in the absorption of the reaction solution at 340 nm over 1 minute was measured for approximately 20 seconds after the addition of RubP with a spectrophotometer and the activity of Rubisco was calculated using the extinction coefficient, 6.22 mM⁻¹ cm⁻¹. In the present study, Rubisco activity was expressed as μ mol of NADPH m⁻² s⁻¹.

To measure the concentration of Rubisco, 50 μ l of the supernatant of the sample was added to the extraction buffer, 100 μ l of Laemmli buffer containing 0.1 M Tris-HCl (pH 6.8), 20% (w/v) glycerol, 4% (w/v) sodium dodecylsulfate (SDS) and 0.004% bromophenol blue, and 20 μ l of 2-mercaptoethanol according to the modified method of Laemmli (1970). After heating at 95°C for 5 minutes, the sample was used for SDS-PAGE. The Rubisco protein was separated by SDS-PAGE using a 1 mm thick polyacrylamide gel (13% separating, 4% stacking) and the amount of Rubisco was calculated based on the density of CBB-stained subunit bands scanned with a gel image analysis system (densitograph, AE-6920MF, ATTO Co., Japan). In the quantification of Rubisco, calibration curve of the protein concentration was made with BSA.

2.5 Measurement of the activity of radical scavenging enzymes

To measure the activities of SOD, CAT and GR, the leaf samples (100 mg FW) stored at -80°C were homogenized to a fine powder in liquid nitrogen with a small amount of quartzose sand. Subsequently, the leaf samples were added to 1 ml of extraction buffer containing 50 mM potassium phosphate (pH 7.8) and 1 mM Na-EDTA, and 20 mg of PVPP. The crude homogenate was centrifuged at 16,000g for 10 minutes at 4°C, while the supernatant of the sample was used in the assay of the activities of SOD, CAT and GR.

The activity of SOD was determined by the methods of McCord and Fridovich (1969). The supernatant (70 μ l) was added to 0.8 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 ml of 1 mM xanthine solution and 10 μ l of 1 mM cytochrome *c* (horse heart, Sigma-Aldrich). After the measurement of blank at 550 nm for 1 minute with a spectrophotometer, 20 μ l of xanthine oxidase solution (from bovine milk, 4.0 units ml⁻¹, Sigma-Aldrich) was added and subsequently, the absorbance was measured for 2 minutes at 550 nm. In the present study, 1 unit of SOD activity was defined as the amount of enzyme required to inhibit ferricytochrome *c* reduction

by 50%.

The activity of CAT was determined by the method of Aebi (1983). The supernatant (20 μ l) was added to 970 μ l of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA. Following the measurement of blank at 240 nm for 1 minute with a spectrophotometer, 10 μ l of 880 mM H₂O₂ was added, whereupon the absorbance was measured for 2 minutes at 240 nm and the activity of CAT was calculated using the extinction coefficient, 40 mM⁻¹ cm⁻¹. In the present study, CAT activity was expressed as mmol of H₂O₂ m⁻² s⁻¹.

The activity of GR was determined by the method of Foyer and Halliwell (1976). The supernatant (50 μ l) was added to 850 μ l of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 50 μ l of 2 mM NADPH. After the measurement of blank at 340 nm for 1 minute with a spectrophotometer, 50 μ l of 10 mM oxidized glutathione (GSSG) was added. Subsequently, the absorbance was measured for 2 minutes at 340 nm. The GR activity was calculated using the extinction coefficient, 6.22 mM⁻¹ cm⁻¹. In the present study, GR activity was expressed as μ mol of NADPH m⁻² s⁻¹.

To measure the activity of APX, leaf samples (50 mg FW), which were stored at -80°C, were homogenized to a fine powder in liquid nitrogen with a small amount of quartzose sand. Subsequently, leaf samples were added to an extraction buffer of 0.5 ml containing 50 mM potassium phosphate (pH 7.0), 1 mM Na-EDTA and 5 mM L-ascorbic acid, and 10 mg of PVPP. The crude homogenate was centrifuged at 16,000g for 10 minutes at 4°C, while the supernatant of the sample was used in the assay of the activity of APX. The activity of APX was determined by the method of Nakano and Asada (1981). The supernatant (20 μ l) was added to 910 μ l of 50 mM potassium phosphate buffer (pH 7.0) and 50 μ l of 5 mM L-ascorbic acid solution. After the measurement of blank at 290 nm for 1 minute with a spectrophotometer, 20 μ l of 50 mM H₂O₂ was added, whereupon the absorbance was measured for 2 minutes at 290 nm and the activity of APX was calculated using the extinction coefficient, 2.8 mM⁻¹ cm⁻¹. In the present study, the activity of APX was expressed as μ mol of ascorbate m⁻² s⁻¹.

When measuring the activities of MDAR and DHAR, the leaf samples (50 mg FW) stored at -80°C were homogenized to a fine powder in liquid nitrogen with a small amount of quartzose sand. Subsequently, these were added to an extraction buffer

of 0.5 ml containing 50 mM Tris-HCl (pH 7.0), 1 mM L-ascorbic acid, 1 mM dithiothreitol, 1 mM EDTA, 1 mM glutathione reduced form and 5 mM magnesium chloride, and 10 mg of PVPP. The crude homogenate was centrifuged at 12,000g for 6 minutes at 4°C and 20,000g for 16 minutes at 4°C and the supernatant of the sample was used in the assay of the activities of MDAR and DHAR.

The activity of MDAR was determined by the method of Hossain *et al.* (1984). The supernatant (100 μ l) was added to 500 μ l of 100 mM Tris-HCl buffer (pH 7.5), 100 μ l of 25 mM L-ascorbic acid solution, 50 μ l of 4 mM NADPH and 249 μ l of deionized water. After the measurement of blank at 340 nm for 1 minute with a spectrophotometer, 1 μ l of ascorbate oxidase (0.15 units μ l⁻¹, Sigma-Aldrich) was added. Subsequently, the absorbance was measured for 3 minutes at 340 nm. The activity of MDAR was calculated using the extinction coefficient, 6.22 mM⁻¹ cm⁻¹. In the present study, MDAR activity was expressed as μ mol of NADPH m⁻² s⁻¹.

The activity of DHAR was determined by the method of Hossain and Asada (1984). The supernatant (2 μ l) was added to 500 μ l of 100 mM potassium phosphate buffer (pH 6.5), 423 μ l of deionized water and 25 μ l of 200 mM reduced glutathione. After the measurement of the blank at 265 nm for 1 minute with a spectrophotometer, 50 μ l of oxidized glutathione was added, whereupon the absorbance was measured for 2 minutes at 265 nm and the DHAR activity was calculated using the extinction coefficient, 14.3 mM⁻¹ cm⁻¹. In the present study, DHAR activity was expressed in the form of μ mol of dehydroascorbate m⁻² s⁻¹.

2.6 Measurement of the concentration and redox state of antioxidants

To measure the concentrations of ascorbate and glutathione, the leaf samples (50 mg FW) stored at -80°C were homogenized to a fine powder in liquid nitrogen with a small amount of quartzose sand. Subsequently, the leaf samples were added to 2.5 ml of 5% (w/v) metaphosphoric acid buffer and 10 mg of PVPP and the crude homogenate was centrifuged at 15,000g for 20 minutes at 4°C. The supernatant of the sample was also used in an assay of the concentrations of ascorbate and glutathione.

The concentration of ascorbate was determined by the modified method of Luwe and Heber (1995). To measure the concentration of reduced ascorbate,

the supernatant (25 μ l) was added to 974 μ l of 100 mM potassium phosphate buffer (pH 7.0). After the measurement of a blank at 265 nm for 1 minute with a spectrophotometer, 1 μ l of ascorbate oxidase (1 unit μ l⁻¹, Sigma-Aldrich) was added, whereupon the absorbance was measured for 2 minutes at 265 nm. To measure the concentration of oxidized ascorbate, the supernatant (50 μ l) was added to 949 μ l of 100 mM potassium phosphate buffer (pH 7.7). Following the measurement of a blank at 265 nm for 1 minute with a spectrophotometer, 1 μ l of 200 mM dithiothreitol (DTT) was added, whereupon the absorbance was measured for 5 minutes at 265 nm. In the present study, the concentration of reduced ascorbate was calculated using the extinction coefficient, 14.3 mM⁻¹ cm⁻¹. The redox state of ascorbate was expressed in the form of the concentration ratio of reduced ascorbate to total ascorbate (the sum of the reduced and oxidized forms of ascorbate).

The concentration of glutathione was determined by the method of Griffith (1980). To measure the concentration of total glutathione (the sum of the reduced and oxidized forms of glutathione), the supernatant (195 μ l) was added to 660 μ l of 125 mM potassium phosphate containing 6.3 mM Na-EDTA, 100 μ l of 6 mM 5, 5'-dithiobis (2-nitrobenzoic acid) and 40 μ l of 5 mM NADPH. After the measurement of blank at 412 nm for 1 minute with a spectrophotometer, 5 μ l of glutathione reductase (from baker's yeast, 1.81 units μ l⁻¹, Sigma-Aldrich) was added. Subsequently, the absorbance was measured for 2 minutes at 412 nm. In the quantification of total glutathione concentration, a calibration curve was constructed, based on the concentrations of the reduced and oxidized forms of glutathione. To measure the concentration of oxidized glutathione, the supernatant (1 ml) was added to 20 μ l of 2-vinylpyridine. Following incubation at 25°C for 40 minutes, the absorbance of the sample was measured at 412 nm for 1 minute with a spectrophotometer and the concentration of reduced glutathione was calculated by subtracting the oxidized glutathione concentration from the total glutathione concentration. The redox state of glutathione was expressed in the form of the ratio of reduced glutathione concentration to the total glutathione concentration.

2.7 Calculation of the O₃ exposure-response relationship

The mean daylight AOT40 of the three chamber replicates in each of the gas treatments from 30 May to

20 September, 2007 was used as the O₃ exposure index in calculating the O₃ exposure-response relationship. The average activity of radical scavenging enzymes in the CF treatment was also used as a reference (100%) in each chamber to calculate the relative enzyme activity for each O₃ treatment. To calculate the coefficient of determination value (R^2) and P value for the slope, a single regression line was obtained from the relationship between the relative activity

of radical scavenging enzymes in the leaves within approximately one month of emergence, and the mean daylight AOT40 of O₃.

2.8 Statistical analyses

Statistical analyses were performed with the SPSS® statistical package. Analysis of variance (ANOVA) was used to test the effects of O₃ and cultivars. When a significant combined effect of O₃ and cultivars was detected, Tukey's HSD test was performed to identify

Table 1. Effects of O₃ on the concentration of chlorophyll and activity and concentration of Rubisco in leaves of rice (*Oryza sativa* L.).

Cultivar	Treatment	Concentration of chlorophyll (g m ⁻²)	Activity of Rubisco (μmol m ⁻² s ⁻¹)	Concentration of Rubisco (g m ⁻²)
Koshihikari (8th leaf)	CF	0.228 (0.001)	7.0 (0.5)	2.05 (0.37)
	60 ppb O ₃	0.213 (0.015)	6.6 (0.3)	1.83 (0.35)
	100 ppb O ₃	0.200 (0.019)	6.0 (0.9)	1.79 (0.14)
Kinuhikari (7th leaf)	CF	0.228 (0.008)	7.3 (0.8)	2.04 (0.28)
	60 ppb O ₃	0.211 (0.014)	6.3 (1.3)	1.80 (0.22)
	100 ppb O ₃	0.200 (0.016)	6.5 (1.1)	1.99 (0.28)
ANOVA	O ₃	*	n.s.	n.s.
	Cultivar	n.s.	n.s.	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.
Koshihikari (10th leaf)	CF	0.269 (0.006)	15.2 (1.1) a	1.80 (0.12)
	60 ppb O ₃	0.248 (0.013)	14.1 (0.7) a	1.60 (0.14)
	100 ppb O ₃	0.226 (0.005)	13.4 (1.1) a	1.44 (0.06)
Kinuhikari (9th leaf)	CF	0.233 (0.014)	13.7 (1.2) a	1.40 (0.05)
	60 ppb O ₃	0.220 (0.009)	13.9 (1.8) a	1.34 (0.07)
	100 ppb O ₃	0.210 (0.009)	13.8 (1.5) a	1.36 (0.06)
ANOVA	O ₃	**	n.s.	n.s.
	Cultivar	*	n.s.	**
	O ₃ ×Cultivar	n.s.	*	n.s.
Koshihikari (Flag leaf)	CF	0.177 (0.002)	5.7 (0.3)	0.76 (0.04)
	60 ppb O ₃	0.177 (0.004)	6.4 (1.1)	0.92 (0.19)
	100 ppb O ₃	0.181 (0.005)	7.8 (1.0)	0.95 (0.06)
Kinuhikari (Flag leaf)	CF	0.179 (0.014)	7.5 (0.6)	1.01 (0.10)
	60 ppb O ₃	0.184 (0.007)	7.9 (0.6)	0.99 (0.06)
	100 ppb O ₃	0.181 (0.012)	8.7 (0.3)	1.09 (0.08)
ANOVA	O ₃	n.s.	*	n.s.
	Cultivar	n.s.	*	**
	O ₃ ×Cultivar	n.s.	n.s.	n.s.

The plants were exposed daily to charcoal-filtered air (CF) or O₃ at 60 ppb or 100 ppb (10:00-17:00) from 30 May to 21 August 2007. Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses.

Two-way ANOVA: * $P<0.05$, ** $P<0.01$, n.s.=not significant

When a significant combined effect of O₃ and cultivar was detected, Turkey's HSD test was performed to identify significant differences among the 6 treatments. Values with different letters are significantly different at $P<0.05$.

Table 2. Effects of O₃ on the activity of radical scavenging enzymes in leaves of rice (*Oryza sativa* L.).

Cultivar	Treatment	SOD (U cm ⁻²)	CAT (mmol m ⁻² s ⁻¹)	APX (μmol m ⁻² s ⁻¹)	MDAR (μmol m ⁻² s ⁻¹)	DHAR (μmol m ⁻² s ⁻¹)	GR (μmol m ⁻² s ⁻¹)
Koshihikari (8th leaf)	CF	14.8 (0.6) a	12.4 (0.5)	13.8 (0.9)	0.16 (0.04)	16.9 (0.8)	1.6 (0.2) a
	60 ppb O ₃	13.9 (0.4) a	11.6 (0.1)	15.2 (2.0)	0.20 (0.01)	12.6 (0.1)	1.6 (0.1) a
	100 ppb O ₃	13.2 (2.5) a	10.0 (1.3)	17.9 (3.0)	0.20 (0.00)	10.1 (1.5)	2.0 (0.2) a
Kinuhihikari (7th leaf)	CF	13.9 (1.9) a	11.9 (0.4)	15.6 (0.9)	0.13 (0.01)	11.2 (0.7)	1.6 (0.1) a
	60 ppb O ₃	13.5 (1.2) a	11.4 (0.9)	17.2 (1.3)	0.19 (0.03)	9.7 (1.0)	1.8 (0.1) a
	100 ppb O ₃	15.5 (2.1) a	8.4 (0.5)	19.4 (2.1)	0.22 (0.04)	8.2 (1.2)	1.9 (0.1) a
ANOVA	O ₃	n.s.	**	**	*	*	*
	Cultivar	n.s.	n.s.	n.s.	n.s.	*	n.s.
	O ₃ ×Cultivar	*	n.s.	n.s.	n.s.	n.s.	*
Koshihikari (10th leaf)	CF	14.6 (0.3)	8.4 (0.5)	13.8 (1.5)	0.07 (0.00)	10.9 (0.8)	1.2 (0.08)
	60 ppb O ₃	14.8 (1.0)	8.6 (0.9)	12.6 (0.8)	0.11 (0.01)	9.7 (1.1)	1.5 (0.22)
	100 ppb O ₃	15.1 (0.6)	8.2 (0.3)	14.7 (1.2)	0.13 (0.01)	7.2 (1.2)	1.6 (0.03)
Kinuhihikari (9th leaf)	CF	13.8 (1.0)	7.0 (0.3)	12.0 (1.0)	0.08 (0.02)	11.2 (1.0)	1.3 (0.06)
	60 ppb O ₃	13.6 (0.5)	7.0 (0.7)	12.6 (0.4)	0.12 (0.04)	8.6 (1.7)	1.3 (0.03)
	100 ppb O ₃	13.7 (1.2)	7.0 (0.5)	14.8 (1.1)	0.15 (0.02)	7.4 (2.2)	1.7 (0.08)
ANOVA	O ₃	n.s.	n.s.	*	*	*	**
	Cultivar	**	*	n.s.	n.s.	n.s.	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Koshihikari (12th leaf)	CF	14.1 (0.7)	6.36 (0.5)	11.7 (0.5)	0.10 (0.02)	13.5 (1.2)	0.8 (0.07)
	60 ppb O ₃	14.3 (0.3)	7.71 (1.0)	12.5 (0.8)	0.09 (0.01)	12.7 (1.8)	0.9 (0.12)
	100 ppb O ₃	13.2 (0.5)	7.39 (0.2)	14.7 (1.5)	0.14 (0.02)	10.6 (0.9)	1.0 (0.09)
Kinuhihikari (11th leaf)	CF	13.3 (1.4)	6.25 (0.8)	10.9 (0.6)	0.13 (0.01)	13.7 (0.5)	1.0 (0.06)
	60 ppb O ₃	12.8 (0.5)	7.37 (0.6)	11.6 (1.3)	0.14 (0.02)	11.6 (1.0)	1.0 (0.09)
	100 ppb O ₃	12.5 (1.9)	6.30 (0.4)	14.2 (0.9)	0.17 (0.02)	8.4 (1.2)	1.3 (0.13)
ANOVA	O ₃	n.s.	*	**	**	***	**
	Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Koshihikari (Flag leaf)	CF	11.2 (0.9)	3.73 (0.2)	8.4 (0.4)	0.06 (0.01)	13.8 (0.4)	0.8 (0.17)
	60 ppb O ₃	11.9 (0.6)	4.04 (0.1)	8.8 (0.7)	0.08 (0.02)	16.6 (2.5)	0.7 (0.11)
	100 ppb O ₃	11.7 (0.2)	4.93 (0.3)	10.6 (1.4)	0.16 (0.02)	15.2 (2.1)	1.1 (0.24)
Kinuhihikari (Flag leaf)	CF	12.5 (0.9)	4.42 (0.4)	11.5 (0.5)	0.09 (0.00)	15.1 (2.1)	1.0 (0.09)
	60 ppb O ₃	11.9 (0.3)	4.98 (0.6)	10.5 (0.5)	0.10 (0.02)	16.3 (1.7)	1.1 (0.04)
	100 ppb O ₃	12.4 (0.2)	5.84 (0.3)	13.6 (0.8)	0.19 (0.02)	18.1 (2.9)	1.4 (0.13)
ANOVA	O ₃	n.s.	*	**	**	n.s.	*
	Cultivar	n.s.	*	*	n.s.	n.s.	*
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

The plants were exposed daily to charcoal-filtered air (CF) or O₃ at 60 ppb or 100 ppb (10:00-17:00) from 30 May to 21 August 2007. Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses.

Two-way ANOVA: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, n.s.=not significant

When a significant combined effect of O₃ and cultivar was detected, Turkey's HSD test was performed to identify significant differences among the 6 treatments. Values with different letters are significantly different at $P<0.05$.

Table 3. Effects of O₃ on the concentrations and redox states of ascorbate and glutathione in leaves of rice (*Oryza sativa* L.).

Cultivar	Treatment	Ascorbate concentration (mmol m ⁻²)	Ascorbate redox state (%)	Glutathione concentration (mmol m ⁻²)	Glutathione redox state (%)
Koshihikari (8th leaf)	CF	0.801 (0.027)	86.9 (2.7)	0.198 (0.015)	84.4 (7.5)
	60 ppb O ₃	0.858 (0.063)	86.5 (1.7)	0.180 (0.007)	85.6 (4.3)
	100 ppb O ₃	0.877 (0.079)	84.5 (4.9)	0.209 (0.056)	82.4 (4.5)
Kinuhikari (7th leaf)	CF	0.824 (0.026)	87.6 (1.2)	0.206 (0.027)	95.4 (1.6)
	60 ppb O ₃	0.863 (0.030)	85.7 (0.3)	0.176 (0.021)	93.8 (3.6)
	100 ppb O ₃	0.817 (0.021)	86.6 (0.1)	0.175 (0.038)	95.5 (2.8)
ANOVA	O ₃	n.s.	n.s.	n.s.	n.s.
	Cultivar	n.s.	n.s.	n.s.	*
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.
Koshihikari (10th leaf)	CF	0.544 (0.019)	79.2 (1.3)	0.221 (0.009)	93.8 (1.7)
	60 ppb O ₃	0.633 (0.056)	84.8 (0.5)	0.226 (0.018)	96.8 (1.8)
	100 ppb O ₃	0.649 (0.024)	85.8 (0.6)	0.232 (0.013)	97.3 (1.2)
Kinuhikari (9th leaf)	CF	0.479 (0.012)	75.9 (1.0)	0.192 (0.016)	95.8 (1.4)
	60 ppb O ₃	0.618 (0.048)	82.0 (4.2)	0.214 (0.019)	97.0 (1.1)
	100 ppb O ₃	0.653 (0.019)	84.7 (0.5)	0.201 (0.023)	95.3 (1.7)
ANOVA	O ₃	**	*	n.s.	n.s.
	Cultivar	n.s.	*	n.s.	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.
Koshihikari (12th leaf)	CF	0.753 (0.118)	81.8 (3.4)	0.244 (0.017)	92.5 (3.0)
	60 ppb O ₃	0.732 (0.079)	78.8 (6.8)	0.232 (0.020)	89.3 (2.9)
	100 ppb O ₃	0.834 (0.041)	84.9 (2.0)	0.250 (0.022)	93.3 (1.0)
Kinuhikari (11th leaf)	CF	0.716 (0.025)	81.6 (2.7)	0.228 (0.044)	92.3 (3.6)
	60 ppb O ₃	0.718 (0.062)	83.0 (2.4)	0.237 (0.046)	96.9 (2.2)
	100 ppb O ₃	0.786 (0.071)	83.4 (2.4)	0.243 (0.092)	96.3 (1.1)
ANOVA	O ₃	n.s.	n.s.	n.s.	n.s.
	Cultivar	*	n.s.	n.s.	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.
Koshihikari (Flag leaf)	CF	0.716 (0.038)	85.5 (0.9)	0.171 (0.008)	89.6 (4.1)
	60 ppb O ₃	0.715 (0.052)	83.8 (4.2)	0.175 (0.013)	84.2 (7.9)
	100 ppb O ₃	0.677 (0.045)	80.8 (1.5)	0.169 (0.025)	91.4 (8.8)
Kinuhikari (Flag leaf)	CF	0.711 (0.044)	84.3 (0.8)	0.188 (0.020)	93.3 (4.9)
	60 ppb O ₃	0.660 (0.125)	84.8 (5.5)	0.196 (0.011)	94.0 (1.9)
	100 ppb O ₃	0.750 (0.070)	84.9 (5.0)	0.216 (0.017)	89.4 (7.3)
ANOVA	O ₃	n.s.	n.s.	n.s.	n.s.
	Cultivar	n.s.	n.s.	*	n.s.
	O ₃ ×Cultivar	n.s.	n.s.	n.s.	n.s.

The plants were exposed daily to charcoal-filtered air (CF) or O₃ at 60 ppb or 100 ppb (10:00-17:00) from 30 May to 21 August 2007. Each value is the mean of 3 chamber replicates, and the standard deviation is shown in parentheses.

Two-way ANOVA: * $P < 0.05$, ** $P < 0.01$, n.s.=not significant

When a significant combined effect of O₃ and cultivar was detected, Turkey's HSD test was performed to identify significant differences among the 6 treatments. Values with different letters are significantly different at $P < 0.05$.

Fig. 1. The relationships between the AOT40 of O₃ and the relative activity of APX, MDAR or GR in the leaves exposed to O₃ to that in the leaves exposed to charcoal-filtered air of the two rice cultivars. The plots show the data pooled among the leaves within approximately one month from the emergence.

significant differences among the 6 treatments.

3. Results

Table 1 shows the effects of O₃ on the concentration of chlorophyll (*a+b*) and the activity and concentration of Rubisco in the leaves of the two rice cultivars. The concentration of chlorophyll in the 8th and 10th leaves of Koshihikari and the 7th and 9th leaves of Kinuhikari was significantly reduced by the exposure to O₃. However, the concentration of chlorophyll in the flag leaf of both cultivars was not significantly affected by the exposure to O₃, while the activity of Rubisco in the 8th and 10th leaves of Koshihikari and the 7th and 9th leaves of Kinuhikari was not significantly affected by exposure to O₃. In contrast, the activity of Rubisco in the flag leaf of both cultivars was significantly increased by the exposure to O₃, although the latter did not significantly affect the concentration of Rubisco in the leaves of both cultivars. There was no significant combined effect of O₃ and cultivar on the concentration of chlorophyll and activity and the concentration of Rubisco in the leaves of both cultivars.

Table 2 indicates the effects of O₃ on the activities of radical scavenging enzymes in the leaves of the two rice cultivars. The activity of SOD was not significantly affected by the exposure to O₃, although the activity of CAT was significantly reduced in the 8th leaf of Koshihikari and the 7th leaf of Kinuhikari, and significantly increased in the 12th and flag leaves of Koshihikari and the 11th and flag leaves of Kinuhikari by the exposure to O₃. The exposure to O₃ significantly increased the activities of APX, MDAR and GR in the leaves of both cultivars. The activity of DHAR in the

8th, 10th and 12th leaves of Koshihikari and the 7th, 9th and 11th leaves of Kinuhikari respectively was significantly reduced by the exposure to O₃, although there was no significant effect of O₃ on the activity of DHAR in the flag leaf of both cultivars, nor any significant combined effect of O₃ and cultivar on the activity of radical scavenging enzymes in the leaves of both cultivars.

Table 3 shows the effects of O₃ on the concentration and redox state of ascorbate or glutathione in the leaves of the two rice cultivars. The concentration and redox state of ascorbate in the 10th leaf of Koshihikari and the 9th leaf of Kinuhikari were significantly increased by the exposure to O₃. In contrast, the exposure to O₃ did not significantly affect the concentration and redox state of ascorbate in the 8th, 12th and flag leaves of Koshihikari and the 7th, 11th and flag leaves of Kinuhikari. On the other hand, the concentration and redox state of glutathione were not significantly affected by the exposure to O₃. There is no significant combined effect of O₃ and cultivar on the concentration of antioxidants in the leaves of both cultivars.

Figure 1 shows the relationships between the AOT40 of O₃ from the leaf emergence to the measurement day of enzyme activity (within approximately 30 days) and the relative activity of APX, MDAR or GR in the leaves exposed to O₃ to that in leaves exposed to charcoal-filtered air of both cultivars. A positive correlation was obtained between the AOT40 of O₃ and relative activity of APX ($R^2=0.586^{***}$), MDAR ($R^2=0.494^{***}$) or GR ($R^2=0.530^{***}$). However, no significant correlations were obtained between the AOT40 of O₃ and the relative activity of SOD, CAT

or DHAR in the leaves exposed to O₃ to that in the leaves exposed to charcoal-filtered air of both cultivars (data not shown).

4. Discussion

In the present study, no significant differences were observed in the effects of O₃ on the concentration of chlorophyll, activity and the concentration of Rubisco, the activities of radical scavenging enzymes and the concentrations of antioxidants between the two cultivars of rice (Tables 1, 2 and 3). However, the effects of O₃ on the concentration of chlorophyll, the activity of Rubisco and the activities of radical scavenging enzymes differed significantly among the leaf positions (Tables 1, 2 and 3). According to previous studies, the concentration of chlorophyll in mature leaves of several crops was reduced by exposure to O₃ (Nie *et al.* 1993; Chernikova *et al.* 2000). The results obtained in the present study indicate that the effects of O₃ on the concentration of chlorophyll in the leaves of Japanese rice differ among the leaf positions, even if the period of exposure to O₃ is the same among the leaves. Therefore, the detoxification capacity of rice leaf to ROS induced by exposure to O₃ may differ among the leaf positions.

The sensitivity of crops to O₃ is influenced by the detoxification capacity of ROS in the leaves (Nouchi 1993; Bender *et al.* 1994; Chernikova *et al.* 2000; Cheng *et al.* 2007). In the present study, the activities of radical scavenging enzymes such as APX, MDAR and GR in the leaves of both cultivars of rice were significantly increased by the exposure to O₃ (Table 2). It was reported that the activities of APX and GR were also increased by exposure to O₃ in rice (Nouchi 1993) in addition to the other environmental stresses such as UV-B radiation (Kim *et al.* 1996). Furthermore, the O₃-induced reduction of DHAR activity and the increase of MDAR activity in the leaves of both cultivars are in agreement with the results obtained from previous studies with *Arabidopsis* (Kubo *et al.* 1995, 1999). According to Kubo *et al.* (1999), the activities of APX and DHAR show an antagonistic response to O₃. When the monodehydroascorbate radical was directly reduced to ascorbate by MDAR, no dehydroascorbate radical was produced (Kubo *et al.* 1999). In the present study, furthermore, a highly positive correlation was obtained between the AOT40 of O₃ and relative activity of APX, MDAR or GR in the leaves exposed to O₃ to that in the leaves exposed

to charcoal-filtered air of the two rice cultivars (Fig. 1). Therefore, APX, MDAR and GR are considered to play a predominant role in the detoxification of O₃ in the leaves of rice.

In the present study, the whole-plant dry mass and grain yield per plant of both cultivars at the final harvest were significantly reduced by the exposure to O₃ (data not shown, Yamaguchi *et al.* 2008). In addition, the relative growth rate of both cultivars during the vegetative period was significantly reduced by the exposure to O₃. During the vegetative period, furthermore, the exposure to O₃ significantly reduced the net assimilation rate and net photosynthetic rate in the two cultivars. These results suggested that the increase in the activity of radical scavenging enzymes such as APX, MDAR and GR was insufficient to prevent the adverse effects of O₃ on the concentration of chlorophyll, photosynthetic capacity, growth and yield of both cultivars. The exposure to O₃ significantly reduced the activity of CAT in the lower leaves, such as the 8th leaf of Koshihikari and the 7th leaf of Kinuhikari (Table 2). This result is coincident with that obtained from the previous study with *Arabidopsis* (Kubo *et al.* 1995). Since H₂O₂ and O₂⁻ inactivate radical scavenging enzymes such as CAT (Bray *et al.* 1974; Kono and Fridovich 1982; Kirkman *et al.* 1987), the O₃-induced accumulation of ROS in the leaves of both cultivars at the early stage of the growth period might lead to a reduction in the activity of CAT.

There were no significant effects of O₃ on the concentrations of Rubisco and chlorophyll, but the activity of Rubisco in the flag leaves of both cultivars of rice was significantly increased by the exposure to O₃ (Table 1). This may be due to the high detoxification capacity to ROS caused by the increase in the activities of CAT, APX, MDAR and GR in the flag leaf (Table 2). The flag leaf may need to acquire high physiological activity in order to provide carbohydrates for developing ear (Wardlaw 1968). Therefore, the results obtained from the present study indicated that the flag leaves of rice possess high levels of detoxification capacity to ROS as compared with the lower leaves. In the present study, the concentration and redox state of ascorbate in the 10th leaf of Koshihikari and the 9th leaf of Kinuhikari were significantly increased by the exposure to O₃ (Table 3). Ascorbate also plays an important role in the detoxification of O₃ (Conkin *et al.* 1996; Cheng *et al.* 2007). Therefore, the O₃-induced increase in the concentration of ascorbate in the leaves of both

cultivars might prevent the O₃-induced reduction in the activity of CAT. Furthermore, the O₃-induced reduction in the concentration of chlorophyll in the 8th leaf of Koshihikari and the 7th leaf of Kinuhikari might be related to the O₃-induced reduction in the activity of CAT in the leaves. In the present study, the difference in the timing of leaf emergence during the growth period changes the extent of the effects of O₃ on the concentration of chlorophyll, even if the period of exposure to O₃ was the same in the leaves. Therefore, the difference in the effects of O₃ on the concentration of chlorophyll among the leaf positions is considered to be mainly attributable to that in the detoxification capacity to ROS induced by the exposure to O₃.

In conclusion, APX, MDAR and GR are considered to play important roles in the antioxidative response of Japanese rice to O₃. However, the detoxification capacity of reactive oxygen species by APX, MDAR and GR is insufficient to prevent the adverse effects of ambient levels of O₃ on the concentration of chlorophyll in the leaves of Japanese rice.

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イネの葉の光合成関連要素と活性酸素消去系に対する オゾンの影響

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要 約

イネの葉における RubP カルボキシラーゼ / オキシゲナーゼ (Rubisco) の活性・濃度, クロロフィル濃度, 活性酸素消去系に対するオゾン (O_3) の影響を調べた。2007 年 5 月 15 日に黒ボク土を詰めたコンテナにコシヒカリとキヌヒカリの幼苗を移植し, チャンバー内で 5 月 30 日から 9 月 20 日までガス処理を行った。ガス処理区として, 活性炭フィルターによって浄化した空気をチャンバー内に導入した浄化空気区, 毎日 10:00 から 17:00 までチャンバー内の O_3 濃度を 60 nl l^{-1} (ppb) に制御した 60 ppb O_3 区および同時刻の O_3 濃度を 100 ppb に制御した 100 ppb O_3 区を設けた。 O_3 暴露によって, Rubisco の活性・濃度に有意な影響は認められなかったが, 葉のクロロフィル濃度は有意に低下した。 O_3 暴露によって両品種のアスコルビン酸ペルオキシダーゼ (APX), モノデ

ヒドロアスコルビン酸レダクターゼ (MDAR) およびグルタチオンレダクターゼ (GR) の活性が有意に増加し, 浄化空気区に対する O_3 暴露区における酵素活性の増加程度と O_3 の AOT40 との間に有意な正の相関が認められた。以上の結果より, 野外濃度レベルの O_3 を長期間にわたって暴露されたイネの葉では APX, MDAR および GR が抗酸化応答において重要な役割を果たすと考えられたが, これらの酵素の活性上昇は O_3 によるクロロフィル濃度の低下を回避するには不十分であることが明らかになった。

キーワード: イネ, オゾン, 活性酸素消去系酵素, 抗酸化物質, RubP カルボキシラーゼ / オキシゲナーゼ (Rubisco)