Effect of Acute O₃ Exposure on Leaf Photosynthesis of Paddy Rice Grown at Elevated CO₂ Concentration

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Leaf gas exchange, chlorophyll fluorescence, and ascorbic acid contents of rice grown at ambient and elevated CO₂ concentrations (400 and 800 cm³ m⁻³, expressed as C⁴⁰⁰ and C⁸⁰⁰) were examined under three O₃ concentrations (0, 0.1 and 0.3 cm³ m⁻³, expressed as O⁰, O^{0.1} and O^{0.3}) to elucidate the effects of acute O₃ exposure. The net photosynthetic rate, maximum and operating quantum efficiencies of photosystem II decreased immediately after O₃ exposure, but these were ameliorated by C⁸⁰⁰. The acclimation of stomatal conductance to elevated CO₂ occurred in rice leaves of C⁸⁰⁰ plants. Therefore, it was anticipated that O₃-induced injury of photosynthesis in the C⁸⁰⁰+O^{0.3} plants was more suppressed than in C⁴⁰⁰→C⁸⁰⁰+O^{0.3} plants (grown at C⁴⁰⁰+O⁰ and received acute C⁸⁰⁰+O^{0.3} exposure). However, no significant difference was found between these plants. The contents of total ascorbic acid, reduced form of ascorbic acid (AA) and dehydroascorbic acid, and redox state of ascorbic acid were unaffected by C⁸⁰⁰ when they were expressed per leaf area. However, the total ascorbic acid and AA contents per fresh and dry mass bases were decreased by C⁸⁰⁰ because the specific leaf weight was increased significantly by C⁸⁰⁰. Therefore, long-term exposure of rice plants to elevated CO₂ might have little effect on antioxidant systems.

Keywords : antioxidant, carbon dioxide, chlorophyll fluorescence, net photosynthesis, *Oryza sativa*, ozone

INTRODUCTION

Although their background levels increase gradually (*i.e.* chronic exposure), photochemical oxidants often generate as much as 0.1–0.2 cm³ m⁻³ in a short period (*i.e.* acute exposure) in rice-growing areas of the Kanto region of Japan during windless, sunny summers (Environ. Improve. Div., Tokyo Metro. Bureau Environ., 2005). Ozone, a major component of photochemical oxidants

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Abbreviations : AA, reduced form of ascorbic acid; C_i, intercellular CO₂ concentration; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; F', steady fluorescence; F₀, minimum fluorescence of dark-adapted state; F_m, maximum fluorescence of dark-adapted state; F_m', maximum fluorescence in the steady state; F_a', difference between F_m' and F'; F_v, variable fluorescence; GR, glutathione reductase; g_s, stomatal conductance; GSH, reduced form of glutathione; LED, light emitting diode; P_N, net photosynthetic rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; R_D, respiration rate; RDS, redox state; ROS, reactive oxygen species; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SLW, specific leaf weight; VPD, vapor pressure deficit

(Cabrera et al., 1988), was estimated to have decreased rice yields up to 10% in the Kanto region in 1981–1985 (Kobayashi, 1999). This deterioration was ascribed to photosynthesis-related processes rather than fertilization at flowering (Imai and Ookoshi, 2011), as was the case of winter wheat (Feng et al., 2007).

Previous studies of rice leaves (Ishioh and Imai, 2005; Imai and Kobori, 2008; Kobayakawa and Imai, 2011a, b) revealed that acute exposure of O_3 ($0.1\text{--}0.3\text{ cm}^3\text{ m}^{-3}$) immediately decreased P_N , g_s , PSII and nitrite reductase activities, and Rubisco content, but it increased the respiration rate (R_D), which subsequently accompanied visible leaf injury with decreased chlorophyll content. However, simultaneously applied elevated CO_2 ameliorated these injuries because of (1) decreased g_s , which limited O_3 flux into leaf mesophyll, and (2) increased the level of intercellular CO_2 concentration (C_i), which promoted P_N , although these were not thorough factors. During fumigation using these gases, endogenous antioxidants such as ascorbic acid and glutathione were inferred as not fully effective to scavenge O_3 effects (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a).

Currently, the atmospheric CO_2 concentration is about $387\text{ cm}^3\text{ m}^{-3}$. Its yearly increment over the past decade was about $2\text{ cm}^3\text{ m}^{-3}$ (WMO WDCGG, 2011). Therefore, fairly high CO_2 concentrations can be reasonably predicted for the future which can be expected to promote the growth and yield of many crop species (Kimball, 1983). To date, experiments conducted for the interaction between O_3 and CO_2 on crop species were simple acute or chronic situations. Therefore, to forecast future interaction between O_3 and CO_2 on rice, it is worthwhile to examine acute exposure of O_3 to rice that is grown at elevated CO_2 . We examined the effect of elevated CO_2 during rice growth on ascorbic acid content as a representative antioxidant in leaves as some reports have described for rice (Nouchi, 1993) and *Arabidopsis thaliana* (Overmyer et al., 2000; Sasaki-Sekimoto et al., 2005) that antioxidants scavenge oxidative stressors including O_3 , and because endogenous antioxidant contents and their redox state reportedly change by elevated CO_2 in soybean (Pritchard et al., 2000) and forage crops (di Toppi et al., 2002).

MATERIALS AND METHODS

Plant materials and gas exposure

On April 13, 2010, seeds of a japonica paddy rice (*Oryza sativa* L. cv. Koshihikari) were sown in plastic pots (diameter $\times$ height = 0.16×0.19 m) containing 2.5 kg of dry soil and 12.5 g of chemical fertilizer (N, P_2O_5 , K_2O = 8, 8, 8%). They were cultivated in natural light gas-exposure chambers (S-2003A, Koito Industries, Yokohama, Japan) at $28/23^\circ\text{C}$ (12-h day/12-h night), 60% RH and 400 or $800\text{ cm}^3\text{ m}^{-3}$ CO_2 (C^{400} or C^{800}) without O_3 (O^0 : $<0.003\text{ cm}^3\text{ m}^{-3}$). Immediately after full expansion of the eighth leaves (Haun index = 8.0; Haun, 1973), 5-h exposure of O_3 [8:00–13:00 (local time) to meet high light conditions] was applied for each level of CO_2 : $C^{400} + O^0$ (control treatment), $C^{800} + O^0$, $C^{400} + O^{0.1}$ or $O^{0.3}$, $C^{800} + O^{0.1}$ or $O^{0.3}$. In addition, acute $C^{800} + O^{0.3}$ exposure ($C^{400} \rightarrow C^{800} + O^{0.3}$) was applied to rice plants grown at $C^{400} + O^0$. Ozone was supplied using a high-voltage O_3 generator with dry air (MO-5A, Ozone System Co., Tokyo, Japan), and CO_2 was supplied from cylinders containing liquid CO_2 . These gases were injected into air that had been filtered through activated charcoal layers. The O_3 and CO_2 concentrations were measured and were computer-controlled using an ultraviolet absorption-type O_3 analyzer (EG-2001F, Ebara Jitsugyo Co., Tokyo, Japan) and an infrared CO_2 analyzer (ZRH, Fuji Electric Systems Co., Tokyo, Japan), respectively. After 5-h gas exposure, the plants were kept at each growth CO_2 concentration without O_3 .

Gas-exchange measurements

Using a portable photosynthesis and transpiration measurement system (LI-6400XT, LI-COR, Lincoln, NE, USA), *in situ* gas-exchange measurements of the eighth, fully unrolled leaves were conducted immediately before (BE: 1–0 h before), immediately after (AE-0: 0.1–1.1 h after), and

1 and 3 d after (AE-1, AE-3) O₃ exposure at C⁴⁰⁰ or C⁸⁰⁰ during 13:00–14:30 (local time) for five replicate plants in each treatment. Environmental conditions within the *LI-COR* cuvette during measurements were set at 28°C leaf temperature, 1.5 kPa VPD, and 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (mixed light from red and blue LEDs). To clarify the acclimation of P_N and g_s to elevated CO₂ (*i.e.* down-regulation of them due to long-term growth at elevated CO₂), gas exchange in plants grown in C⁸⁰⁰ and C⁴⁰⁰ conditions was measured at C⁸⁰⁰. Then the acclimatory effect was calculated using the method described by Bunce (2001) as the ratio of P_N (or g_s) measured at C⁸⁰⁰ of the C⁸⁰⁰-grown plant to that of the C⁴⁰⁰-grown plant.

Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM, Heinz Walz, Effeltrich, Germany), the chlorophyll fluorescence of the eighth, fully unrolled leaves was measured simultaneously with the gas exchange measurements for five other replicate plants. The respective chlorophyll fluorescence parameters were obtained by application of 0.2, 7,000, and 1,400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of measuring light, saturating pulse (0.8 s flash), and actinic light. Before fluorescence measurements, leaves were kept in the dark for 10 min (to prevent recovery from O₃ injury; Sonoike, 2009). Then the minimum (F_0) and maximum (F_m) fluorescence were determined by irradiating the measuring light and saturating pulses, respectively. Thereafter, the steady fluorescence (F') and maximum fluorescence in the steady state (F_m') were determined under actinic light irradiation. The maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies of PSII were obtained using the following equations (Baker, 2008; Sonoike, 2009).

$$\begin{aligned} F_v/F_m &= (F_m - F_0)/F_m \\ F_q'/F_m' &= (F_m' - F')/F_m' \end{aligned}$$

Ascorbic acid measurements

Immediately before gas exposure, five fully-unrolled eighth leaves developed at C⁴⁰⁰ and C⁸⁰⁰ were sampled and used for measurements of ascorbic acid (reduced form, AA; oxidized form, DHA). Immediately after measurements of fresh mass and leaf area, leaves were frozen in liquid N₂ and ground with a mortar and pestle by adding metaphosphoric acid to obtain leaf extracts. Then they were determined by the hydrazine method (Fujita and Yamada, 2006) with a spectrophotometer (Ubest-30, JASCO Corp., Tokyo, Japan), using L(+)-ascorbic acid (Kanto Chemical Co., Inc., Tokyo, Japan) as a standard reagent. This method uses a color reaction (540 nm) between 2, 4-dinitrophenylhydrazine and sulfuric acid to assay DHA. When sodium 2, 6-dichloroindophenol is added to the sample solution beforehand, the AA is transformed to DHA and total ascorbic acid is obtainable. The content of AA is calculated by subtracting DHA from total ascorbic acid. The redox state (RDS) of ascorbic acid was calculated as AA/(AA + DHA). Using a separate set of plants, the area of the eighth leaf was measured. Its dry mass was obtained after oven-drying at 80°C for 48 h.

Statistical analysis

Analysis of variance (ANOVA) was conducted using software (Excel Statistics 2010 for Windows, Social Survey Research Information Co., Tokyo, Japan). Appropriate standard errors of the means were calculated for presentation with a line diagram. The significance of the treatment effect was determined using *F*-tests.

RESULTS

Acclimation of photosynthesis parameters to elevated CO₂

The P_N and g_s in the C⁴⁰⁰+O⁰ and C⁸⁰⁰+O⁰ plants immediately before O₃ exposure (BE) are shown in Table 1. Responses of P_N and g_s of the C⁸⁰⁰+O⁰ plants were lower than those of the C⁴⁰⁰+O⁰ plants under both CO₂ concentrations measured (C⁴⁰⁰ and C⁸⁰⁰). Therefore, the acclimatory

Table 1 Effects of increased CO₂ concentration on net photosynthetic rate (P_N), stomatal conductance (g_s), and their acclimation in rice plants. The acclimatory effect is the ratio of P_N or g_s measured at 800 cm³ m⁻³ CO₂ in the C⁸⁰⁰+O⁰ plant to that in the C⁴⁰⁰+O⁰ plant. *** P <0.001, n.s.=not significant, as inferred using two-way ANOVA.

Growth CO ₂ [cm ³ m ⁻³]	Measured CO ₂ [cm ³ m ⁻³]	P_N [μmol (CO ₂) m ⁻² s ⁻¹]	g_s [mol (CO ₂) m ⁻² s ⁻¹]	Acclimatory effect	
				P_N	g_s
C ⁴⁰⁰	C ⁴⁰⁰	28.42±0.42	0.711±0.031	0.91	0.73
	C ⁸⁰⁰	36.71±0.53	0.608±0.014		
C ⁸⁰⁰	C ⁴⁰⁰	25.91±0.55	0.504±0.023		
	C ⁸⁰⁰	33.23±0.76	0.441±0.022		
ANOVA	Factor				
	G _{CO₂}	***	***		
	M _{CO₂}	***	***		
	G _{CO₂} ×G _{CO₂}	n.s.	n.s.		

effect of elevated CO₂ during growth on P_N and g_s was calculated respectively as 0.91 and 0.73. Clear direct effects of elevated CO₂ on P_N (+28 to +29%) and g_s (−12 to −14%) were found but no interaction was found between the CO₂ concentration during plant growth and gas exchange measurements.

Effects of O₃ exposure on photosynthesis in rice grown at ambient or elevated CO₂

At AE-0, the P_N of the C⁴⁰⁰+O^{0.1} and C⁴⁰⁰+O^{0.3} plants respectively decreased to 64% and 34% of those at BE (Fig. 1a). At AE-3, the O₃-inhibition recovered fully in the C⁴⁰⁰+O^{0.1} plants but remained in the C⁴⁰⁰+O^{0.3} plants (71% of BE) (Fig. 1a). In addition, the g_s of the C⁴⁰⁰+O^{0.1} and C⁴⁰⁰+O^{0.3} plants at AE-0 respectively decreased to 41% and 22% of those at BE. At AE-3, the g_s of the C⁴⁰⁰+O^{0.1} plants fully recovered from O₃-inhibition (99% of BE) but the C⁴⁰⁰+O^{0.3} plants did not (78% of BE) (Fig. 1b). In C⁴⁰⁰→C⁸⁰⁰+O^{0.3} plants, the P_N and g_s at AE-0 respectively decreased to 54% and 37% of those at BE, but the inhibition was less than that in C⁴⁰⁰+O^{0.3} plants (Fig. 1a, 1b).

The F_v/F_m of the C⁴⁰⁰+O^{0.1} and C⁴⁰⁰+O^{0.3} plants at AE-0 respectively decreased to 91% and 78% of those at BE. At AE-1, the C⁴⁰⁰+O^{0.1} plants recovered completely, but the C⁴⁰⁰+O^{0.3} plants showed an inhibition at AE-3, although the difference from the control plants was not significant (Fig. 1e; Table 2). The F_q'/F_m' of the C⁴⁰⁰+O^{0.1} and C⁴⁰⁰+O^{0.3} plants at AE-0 respectively decreased to 85% and 77% of those at BE. At AE-1, the C⁴⁰⁰+O^{0.1} plants recovered but the C⁴⁰⁰+O^{0.3} plants remained low at AE-3 (83% of BE), although the difference from the control plants was not significant (Fig. 1f; Table 2). The O₃-inhibition of the F_v/F_m and F_q'/F_m' in the C⁴⁰⁰→C⁸⁰⁰+O^{0.3} plants at AE-0 was less than that in the C⁴⁰⁰+O^{0.3} plants (87% and 82% of BE, respectively). At AE-1, the statistical difference between the C⁴⁰⁰→C⁸⁰⁰+O^{0.3} plants and the control plants diminished (Fig. 1e, 1f; Table 2).

The P_N in the C⁸⁰⁰+O^{0.1} plants was unaffected by O₃. At AE-0, the P_N of the C⁸⁰⁰+O^{0.3} plants decreased to 53% of that at BE. The P_N recovered respectively to 72% and 88% at AE-1 and AE-3 (Fig. 1c). The g_s of the C⁸⁰⁰+O^{0.1} and C⁸⁰⁰+O^{0.3} plants at AE-0 respectively decreased to 73% and 61% of that at BE. At AE-1, it recovered in the C⁸⁰⁰+O^{0.1} plants, but it remained low in C⁸⁰⁰+O^{0.3} plants (72% of BE) (Fig. 1d).

The F_v/F_m in the C⁸⁰⁰+O^{0.1} plants was unaffected by O₃, but that in the C⁸⁰⁰+O^{0.3} plants at AE-0 tended to decrease (86% of BE). However, it was not significantly different from the control plants at AE-1 and AE-3 (Fig. 1g; Table 2). The F_q'/F_m' in the C⁸⁰⁰+O^{0.1} plants was unaffected by gas exposure, but that in the C⁸⁰⁰+O^{0.3} plants at AE-0 tended to decrease (84% of BE). It was not significantly different from the control plants at AE-1 and AE-3 (Fig. 1h; Table 2).

Results show that there are significant effects of O₃, CO₂, or O₃×CO₂ on P_N or g_s throughout AE-0 to AE-3. However, such trends are ambiguous in F_v/F_m and F_q'/F_m' (Table 2).

Fig. 1 Effects of acute O₃ exposure on net photosynthetic rate (P_N), stomatal conductance (g_s), maximum efficiency (F_v/F_m) and operating efficiency (F_q'/F_m') of photosystem II in rice plants growing at ambient (C^{400}) or elevated (C^{800}) CO₂ concentration. Vertical bars represent standard errors of the means ($n=5$). BE, AE-0, AE-1, and AE-3 respectively denote data obtained immediately before, immediately after, and 1, and 3 d after O₃ exposure. ●, $C^{400} + O^0$; ○, $C^{800} + O^0$; ▲, $C^{400} + O^{0.1}$; △, $C^{800} + O^{0.1}$; ■, $C^{400} + O^{0.3}$; □, $C^{800} + O^{0.3}$; ◆, $C^{400} \rightarrow C^{800} + O^{0.3}$.

Effect of growth CO₂ concentration on ascorbic acid content

The contents of total ascorbic acid, AA and DHA, and RDS of the eighth leaves were not affected significantly by C^{800} during growth when they were expressed on a per leaf area basis. However, the contents of total ascorbic acid and AA per fresh and dry mass bases were decreased by C^{800} because the specific leaf weight (SLW), which reflected the leaf thickness, was increased

Table 2 Statistical analyses of the effects of O₃ and CO₂ on gas exchange and chlorophyll fluorescence parameters in rice leaves shown in Fig. 1. AE-0, AE-1, and AE-3 respectively indicate 0, 1, and 3 d after gas exposure. * $P < 0.05$, *** $P < 0.001$, n.s.=not significant, as inferred using two-way ANOVA.

Time	Factor	P_N	g_s	F_q/F_m	F_q'/F_m'
AE-0	O ₃	***	***	***	***
	CO ₂	***	n.s.	*	n.s.
	O ₃ × CO ₂	***	***	*	n.s.
AE-1	O ₃	***	***	***	*
	CO ₂	***	***	***	n.s.
AE-3	O ₃ × CO ₂	n.s.	***	n.s.	n.s.
	O ₃	***	***	n.s.	n.s.
	CO ₂	***	***	n.s.	n.s.
	O ₃ × CO ₂	n.s.	***	n.s.	n.s.

Table 3 Effects of growth CO₂ concentration on reduced form (AA), oxidized form (DHA), and redox state (RDS) of ascorbic acid in eighth leaves of rice plants. In each column, means ($n=5$) followed by the same letter are not significantly different at $P < 0.05$ level as inferred from results of one-way ANOVA.

	CO ₂ [cm ³ m ⁻³]	Total	AA	DHA	RDS
Per fresh mass	C ⁴⁰⁰	9.94a	7.90a	2.04a	0.795a
[mmol kg ⁻¹]	C ⁸⁰⁰	8.72b	6.65b	2.07a	0.762a
Per dry mass	C ⁴⁰⁰	34.80a	27.71a	7.17a	0.795a
[mmol kg ⁻¹]	C ⁸⁰⁰	27.54b	21.01b	6.54a	0.762a
Per leaf area	C ⁴⁰⁰	1.36a	1.08a	0.28a	0.795a
[mmol m ⁻²]	C ⁸⁰⁰	1.24a	0.95a	0.29a	0.762a

significantly ($P < 0.05$) by C⁸⁰⁰: 3.9 and 4.5 g m⁻² at C⁴⁰⁰ and C⁸⁰⁰, respectively (Table 3).

DISCUSSION

Kobayakawa and Imai (2011a) observed that a short term, acute exposure of O₃ (O^{0.1} and O^{0.3}) inhibited P_N and F_q'/F_m' , but these inhibitions were ameliorated by concomitant, elevated CO₂ (C⁸⁰⁰). The causal factors of amelioration were ascribed to stomatal closure induced by elevated CO₂, which limited the intake of O₃ into leaf mesophyll (McKee et al., 2000; Płazek et al., 2000; Booker and Fiscus, 2005), and to elevated C_i, which promoted P_N (Booker and Fiscus, 2005; Imai and Kobori, 2008). Consistent with the experiments described above, our present results confirmed that major photosynthetic parameters (P_N , g_s and F_q'/F_m') were inhibited by acute exposure of O^{0.1} and O^{0.3} in ambient CO₂-grown (C⁴⁰⁰) plants, but the inhibition was smaller in elevated CO₂-grown (C⁸⁰⁰) plants (Fig. 1).

Because g_s (*i.e.* stomatal aperture) was a dominant factor for the O₃-induced inhibition of P_N , the elevated CO₂-grown plants should be less inhibited than ambient CO₂-grown plants because, in this study, the g_s of plants showed clear acclimation (*i.e.* lower stomatal aperture) to C⁸⁰⁰ (Table 1). However, the amelioration of O₃-inhibition by C⁸⁰⁰ in the C⁸⁰⁰ + O^{0.3} and C⁴⁰⁰ → C⁸⁰⁰ + O^{0.3} plants was similar (Fig. 1a, 1c). Consequently, the limitation of O₃ uptake through stomata cannot fully explain the O₃-induced inhibition of P_N and its amelioration by elevated CO₂, as shown by the P_N -C_i relations measured under different O₃ and CO₂ concentrations. O^{0.3} caused irreversible damage to mesophyll function, but O^{0.1} did not (Imai and Kobori, 2008). Such a limited role of elevated CO₂ to the restriction of O₃ uptake through stomata has been inferred for spring wheat (Mulholland et al., 1997). However, causal factors of the amelioration by elevated CO₂ other than reduced g_s and elevated C_i are not clearly identified, as described previously.

Recently, Gillespie et al. (2011) conducted experiments on the effect of acute O₃ (0.2 cm³ m⁻³) exposure of soybean plants grown at elevated CO₂ (650 cm³ m⁻³) finding that the sensitivity of plants to acute O₃ exposure was increased because of decreased antioxidant capacity. In relation to this, they observed unchanged total phenolic content that might relate to scavenge O₃ stress. Previously, Peñuelas et al. (1997) elucidated from a literature review of 17 plant species that the production of carbon-based-secondary compounds (phenolics and terpenoids) increased with elevation of CO₂ (350, 700, and 1,050 cm³ m⁻³). Ghasemzadeh and Jaafar (2011) grew ginger plants at ambient and elevated CO₂ (400 vs. 800 cm³ m⁻³) and observed increased levels of total flavonoids, total phenolics, and antioxidant activity at elevated CO₂. In addition, Pritchard et al. (2000) grew two soybean genotypes (resistant vs. susceptible to frogeye leaf spot disease) at ambient and elevated CO₂ (365 vs. 720 cm³ m⁻³) and reported that activities of constitutive antioxidative enzymes such as superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, glutathione peroxidase, and glutathione reductase (GR) were decreased in both genotypes at elevated CO₂. However, those of dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase were increased in susceptible genotype but were decreased in tolerant genotype at elevated CO₂. Schwanz and Polle (2001) reported that elevated CO₂ (700 and 1,200 cm³ m⁻³) ameliorated the decreases in antioxidant protection capacity and lipid peroxidation in leaves of *Quercus robur* and *Pinus pinaster* by drought stress. di Toppi et al. (2002) grew two crop species at elevated CO₂ (ambient vs. 560 cm³ m⁻³) and observed remarkable variation in ascorbate and glutathione pool sizes and related enzyme activities: *Dactylis glomerata* showed increased DHAR, AA, GR, and a reduced form of glutathione (GSH), but *Trifolium repens* showed increased DHAR and GR, and decreased AA and GSH, at elevated CO₂. Therefore, it is difficult to draw a general scheme of the antioxidative responses of plants to elevated CO₂, probably because of species and/or genotype specific reasons. Regarding responses of plants to elevated CO₂ and O₃, Gillespie et al. (2011) claimed that these two factors differently affected the antioxidant system.

To clarify whether ascorbic acid is actually effective to scavenge O₃-stress in rice leaves or not, we examined the effect of long-term CO₂ enrichment on ascorbic acid according to some discrepancies (Nouchi, 1993; Imai and Kobori, 2008; Kobayakawa and Imai, 2011a). Table 3 shows that elevated CO₂ concentration during growth did not affect the synthesis of ascorbic acid when expressed on leaf area basis, but its apparent content decreased when expressed on fresh and dry mass bases because of increased SLW. However, although the RDS of ascorbic acid was not significantly different, the leaves developed at C⁸⁰⁰ showed slightly lower values than that at C⁴⁰⁰, suggesting that the reactive oxygen species (ROS) was increased somewhat by elevated CO₂. Cheeseman (2006) reported that elevated CO₂ (550 cm³ m⁻³) increased the H₂O₂ level in soybean leaves by 42% compared to that at ambient CO₂ (375 cm³ m⁻³). In addition, Qiu et al. (2008) described that the protein carbonylation, a marker of oxidative stress, increased in *Arabidopsis thaliana* and soybean, which were grown at elevated CO₂. Because no such information exists for rice plants, we must examine whether the ROS was increased or not at elevated CO₂, as well as the phenolics.

In summary, the photosynthesis parameters of elevated CO₂-grown rice plants were inhibited by acute O₃ exposure, but the inhibition was less than that of ambient CO₂-grown plants when compared at each growth CO₂ concentration. However, the ascorbic acid content was almost unchanged during the growth at elevated CO₂. Therefore, it did not explain the amelioration by elevated CO₂.

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