Relation between O₃-inhibition of Photosynthesis and Salicylic Acid in Paddy Rice Grown with Different CO₂ Concentrations

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This study was conducted to evaluate the role of salicylic acid (SA) on acute ozone (O₃: 0, 0.1, and 0.3 cm³ m⁻³; O⁰, O^{0.1} and O^{0.3}, respectively)-induced photosynthetic inhibition of paddy rice leaves given different atmospheric carbon dioxide concentrations (CO₂: 400 and 800 cm³ m⁻³; C⁴⁰⁰ and C⁸⁰⁰, respectively). Salicylic acid solutions (0, 0.1, and 1 mM; S⁰, S^{0.1}, and S¹, respectively) were applied as a pretreatment one day before O₃ exposure. Gas exchange, chlorophyll fluorescence, and ascorbic acid were measured immediately before (BE), immediately after (AE-0), and 1 d and 3 d after (AE-1, AE-3) 5-h O₃ exposure. The photosynthesis-related parameters, total ascorbic acid, and redox state of ascorbic acid (RDS) were decreased by O₃ exposure. The O₃-induced reduction of P_N , ascorbic acid content, and its RDS were ameliorated by C⁸⁰⁰. Salicylic acid ameliorated the O₃-inhibition of net photosynthetic rate (P_N) slightly, and O₃-induced depletion of total ascorbic acid and RDS substantially. SA did not increase P_N in non-treated leaves (O⁰ plants). However, O₃ exposure elevated the level of endogenous SA. These results show that SA plays a vital role in the defense response to acute O₃ exposure in paddy rice. Effects of SA on O₃-inhibition of PSII and ascorbic acid content were unaffected by elevated CO₂ (C⁸⁰⁰).

Keywords : antioxidant, carbon dioxide, *Oryza sativa*, ozone, photosynthesis, salicylic acid

INTRODUCTION

In the Kanto region of Japan, 10–20 photochemical oxidant warnings are issued every rice growth season. Hourly peak values of photochemical oxidants sometimes approach 0.2 cm³ m⁻³ (Environ. Improv. Div., Tokyo Metro. Bureau Environ., 2005). Of photochemical oxidant components, 90% or more is O₃ (Cabrera et al., 1988; Nouchi, 2001). When exposed to O₃, rice plants suffer damage: inhibition of net photosynthetic rate (P_N), stomatal conductance (g_s) and PSII (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a, 2012a), decreased ribulose 1,5-bisphosphate carboxylase/oxygenase (Ishioh and Imai, 2005), chlorophyll and carotenoid (Rai and Agrawal, 2008) contents and nitrite reductase activity (Kobayakawa and Imai, 2011b), in addition to visible leaf-related symptoms (Imai and Kobori, 2008) and breakdown of the cellular ultrastructure (Toyama et al., 1989). Moreover, O₃ suppresses growth (Imai and Ookoshi, 2011), alters photoassimilate partitioning (Nouchi et al., 1995), and decreases grain yields (Reid and Fiscus, 2008; Imai and Ookoshi, 2011).

Atmospheric CO₂ concentrations have increased worldwide from a pre-industrial level of about 280 cm³ m⁻³ to the current level of 391 cm³ m⁻³. This trend is ex-

pected to continue unless human activities are curtailed substantially (WMO WDCGG, 2013). Under elevated CO₂, g_s generally decreases. Then the O₃-inhibition decreases because of the limited inflow of O₃ through stomata (Booker and Fiscus, 2005; Imai and Kobori, 2008). In rice plants, O₃-inhibition of photosynthesis is ameliorated by elevated CO₂ (Imai and Kobori, 2008; Kobayakawa and Imai, 2011a, 2012a). Similar responses have been found in other plant species (Vandermeiren et al., 2009).

Recently, plant hormones such as jasmonic acid (JA), salicylic acid (SA) (Janda et al., 2007), and ethylene (Druege, 2006) have been recognized as important signals in O₃ stress (Kangasjärvi et al., 2005; Rao and Davis, 2001). In paddy rice, Kobayakawa and Imai (2012b) found that methyl jasmonate (MeJA) application ameliorated O₃-inhibition of P_N by inducing stomatal closure and by increasing antioxidant capacity. After repeated examinations of *Arabidopsis* (Overmyer et al., 2000; Kanna et al., 2003) and tobacco (Örvar et al., 1997), the jasmonates are believed to play an important role in ameliorating O₃ injury. Reportedly, SA is related to plant processes such as thermogenesis, seed germination, respiration, leaf senescence, and disease resistance (An and Mou, 2011). Deteriorative effects of stresses such as heavy metals, salinity, and temperature are ameliorated by exogenously applied SA (Hayat et al., 2010). Several reports describe that

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Abbreviations: AA, reduced form of ascorbic acid; CO₂, carbon dioxide; DHA, dehydroascorbic acid; F_q'/F_m' , operating quantum efficiency; F_v/F_m , maximum quantum efficiency; g_s , stomatal conductance; JA, jasmonic acid; MA, mandelic acid; MeJA, methyl jasmonate; O₃, ozone; P_N , net photosynthetic rate; PPFD, photosynthetic photon flux density; PSII, photosystem II; RDS, redox state of ascorbic acid; SA, salicylic acid; VPD, vapor pressure deficit

O₃ exposure increases endogenous SA in *Arabidopsis* (Sharma et al., 1996; Rao et al., 2000), tobacco (Yalpani et al., 1994), and poplar (Koch et al., 2000). Yoshida et al. (2009b) found for *Arabidopsis* that the O₃ injury of double mutant of *vtc1* (lacking ascorbic acid biosynthesis) and *sid2* (lacking salicylic acid biosynthesis) was severer than that of single mutants of *vtc1*. In fact, SA plays a vital role in cell death. Without SA, active programmed cell death is not initiated in O₃-exposed plants (Kangasjärvi et al., 2005). Consequently, SA is believed to be related to the O₃ response of several model plants, although details of its role remain unclear.

This study evaluated the role of SA in defending against acute O₃-inhibition of P_N in paddy rice grown under different CO₂ concentrations by application of SA exogenously and by quantifying endogenous SA.

MATERIALS AND METHODS

Plant materials, gas exposure treatments, and SA applications

In April (Exp. 1) and August (Exp. 2) of 2012, japonica rice (*Oryza sativa* L. cv. Koshihikari) seeds were sown in Wagner pots (1/5000 a) filled with 2.1 kg of dry soil and 12.5 g of chemical fertilizer (N, P₂O₅, K₂O=8, 8, 8%). Plants were raised in natural-light growth chambers (width × depth × height=2 m × 2 m × 1.9 m, S-2003A; Koito Industries, Ltd., Yokohama, Japan) at 28/23°C (12-h day/12-h night), 60% RH and 400 or 800 cm³ m⁻³ CO₂ (C⁴⁰⁰ or C⁸⁰⁰) without O₃. In Exp. 1, immediately after full expansion of the eighth leaves (Haun index=8.0; Haun, 1973), 0.3 mL SA (Wako Pure Chemical Industries Ltd., Oosaka, Japan) solutions [0 (S⁰), 0.1 (S^{0.1}), and 1 (S¹) mM in 0.5% Tween 20 solution] were applied to the eighth leaves using soft brushes. One day after SA application (Exp. 1) or immediately after full expansion of the eighth leaves (Exp. 2), rice plants were exposed to 0, 0.1, and 0.3 cm³ m⁻³ O₃ (expressed respectively as O⁰, O^{0.1}, and O^{0.3}) during 5-h local daytime (08:00–13:00) under growth CO₂ concentrations. Ozone was supplied using a high-voltage O₃ generator with dry air (ED-OG-R6; Ecodesign Inc., Ogawa, Saitama, Japan), and CO₂ was supplied from cylinders containing liquid CO₂. These gases were injected into air that had been filtered through activated charcoal layers. The O₃ and CO₂ concentrations were measured and computer-controlled using an ultraviolet absorption-type O₃ analyzer (EG-2001F; Ebara Jitsugyo Co., Ltd., Tokyo, Japan) and an infrared CO₂ analyzer (ZRH; Fuji Electric Systems Co., Ltd., Tokyo, Japan).

Gas exchange measurements

In Exp. 1, gas-exchange measurements of the attached, eighth leaves at the middle portion during 13:00–14:30 (local time) were conducted immediately before (BE: 1–0 h before), immediately after (AE-0: 0.1–1.1 h after), 1 d and 3 d after (AE-1, AE-3) O₃ exposures at C⁴⁰⁰ or C⁸⁰⁰ for four replicate plants in each treatment using a portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA). Environmental conditions within the Li-Cor cuvette during measurements were set at 28°C leaf

temperature, 1.5 kPa VPD, and 1,500 μmol m⁻² s⁻¹ PPFD (mixed light from red and blue LEDs) under growth CO₂ concentrations.

Chlorophyll fluorescence measurements

In both Exp. 1 and Exp. 2, chlorophyll fluorescence of the eighth leaves was measured at 28°C simultaneously with gas-exchange measurements for four replicate plants using a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany). The respective chlorophyll fluorescence parameters were obtained by application of 0.2, 7,000, and 1,400 μmol m⁻² s⁻¹ of measuring light, saturation pulse (0.8 s flash), and actinic light. Before fluorescence measurements, the leaf was kept in darkness for 10 min. This dark period was taken to intercept the recovery of leaves from O₃ injury to examine potential values. Sonoike (2009) reported that the effect of acute stress recovered with elapsed time although, without stress, the dark acclimation period is 30 min or longer. The minimum (F₀) and maximum (F_m) fluorescence were determined, respectively, by irradiating the measuring light and saturation pulses. 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).

$$F_v/F_m = (F_m - F_0)/F_m$$

$$F_q'/F_m' = (F_m' - F')/F_m'$$

Ascorbic acid measurements

In Exp. 1, four eighth leaves for each treatment were sampled and used for the measurements of ascorbic acid (reduced form, AA; oxidized form, DHA) contents simultaneously with gas exchange measurements. Immediately after measurements of the fresh weight and leaf area, the leaves were frozen in liquid N₂ and were ground with a mortar and pestle by adding metaphosphoric acid to obtain leaf extracts. Then they were determined using the hydrazine method (Fujita and Yamada, 2006) with a spectrophotometer (Ubest-30; Jasco Corp., Tokyo, Japan), with L(+)-ascorbic acid (Kanto Chemical Co., Inc., Tokyo, Japan) as the 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, AA is transformed to DHA and the total ascorbic acid is obtainable. The AA content was calculated using subtraction of DHA from the total ascorbic acid. The redox state (RDS) of ascorbic acid was calculated as AA/(AA+DHA).

Salicylic acid measurements

In Exp. 2, four eighth leaves for each treatment were sampled and used for measurements of SA simultaneously with chlorophyll fluorescence measurements. Immediately after measurements of the fresh weight, leaves were frozen in liquid N₂ and were kept at -80°C in a deep freezer (ESL-70A; Ebara Inc., Tokyo, Japan) until assay. The SA content was determined as described by Coquoz et al. (1998) and Deng et al. (2003) with a slight modification. The leaf sample (ca. 1 g FW) was homogenized in 3 mL

chloroform/methanol, 9:1 (v/v) with (+)-mandelic acid (MA; Wako Pure Chemical Industries Ltd., Osaka, Japan) as an internal standard using a homogenizer (*T10 Basic* + *S10N-10G*; IKA®-Werke GmbH & Co. KG, Staufen, Germany). The organic phase extract was dried at 25°C using a pressured gas blowing concentrator (*MG-2200* + *S-024*; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with N₂. The sample was dissolved into the solution of 100 µL anhydrous pyridine and 100 µL *Sylon BT* reagent ([N, O-bis(trimethylsilyl) acetamide]: trimethylchlorosilane = 5 : 1 (v/v); Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and was derivatized for 60 min at 80°C. Then, the solution was re-evaporated to dryness at 25°C under gaseous N₂. Thereafter, the dried sample was re-dissolved in 100 µL methanol and was injected into a gas chromatography — mass spectrometer (*GCMS-QP2010*; Shimadzu Corp., Kyoto, Japan). Separation was conducted on a capillary column (30 m × 0.25 mm × 0.25 µm, *Rtx®-5 ms*; Restek Corp., Pennsylvania, U.S.) using He as a carrier gas. The ion source and interface temperatures were maintained, respectively, at 260°C and 250°C. The column temperature was held at 140°C for 2 min and then elevated by 4°C min⁻¹ to 290°C. The retention times of trimethylsilyl derivatives of SA and MA (Fig. 1) were, respectively, 7.048

and 6.400 min. Ion strength for SA was monitored by m/z 267 and 149. For MA, it was monitored by m/z 179 and 147. The SA concentration in the original extract was determined from the ratio of peak areas of m/z 267 (SA) / 179 (MA).

Statistical analysis

All data were subjected to three-way analysis of variance (ANOVA) using a software package (*Excel Statistics 2010 for Windows*; Social Survey Research Information Co., Ltd., Tokyo, Japan). The significance among treatments in each time interval was determined using the least significant difference test (LSD, $P \leq 0.05$).

RESULTS

Effect of SA on O₃-inhibition of photosynthesis under ambient CO₂ (Exp. 1)

Photosynthesis-related parameters in the O⁰ plants were unaffected by SA application during AE-0 to AE-3 (Fig. 2). At AE-0, P_N in the O^{0.1} + C⁴⁰⁰ + S⁰ and O^{0.3} + C⁴⁰⁰ + S⁰ plants were respectively 54% and 39% of those at BE. Thereafter, the plants recovered from O₃-inhibition. At AE-3, it had recovered substantially in the O^{0.1} + C⁴⁰⁰ + S⁰ plants (−12% of BE) but persisted in the O^{0.3} + C⁴⁰⁰ + S⁰

Fig. 1 Mass spectra of the trimethylsilyl derivatives of salicylic acid (a) and mandelic acid (b).

Fig. 2 Effects of O₃ and salicylic acid (SA) on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_v'/F_m') quantum efficiency of photosystem II at ambient CO₂ concentration in rice leaves (Exp. 1): vertical bars represent LSD ($P \leq 0.05$); BE, AE-0, AE-1, AE-3 — before, immediately after, 1 d and 3 d after gas exposure; O⁰, O^{0.1}, O^{0.3} — 0, 0.1, 0.3 cm³ m⁻³ O₃; C⁴⁰⁰ — 400 cm³ m⁻³ CO₂; S⁰, S^{0.1}, S¹ — 0, 0.1, 1 mM SA; ●, O⁰ + C⁴⁰⁰ + S⁰; ●, O⁰ + C⁴⁰⁰ + S^{0.1}; ○, O⁰ + C⁴⁰⁰ + S¹; ▲, O^{0.1} + C⁴⁰⁰ + S⁰; ▲, O^{0.1} + C⁴⁰⁰ + S^{0.1}; △, O^{0.1} + C⁴⁰⁰ + S¹; ■, O^{0.3} + C⁴⁰⁰ + S⁰; ■, O^{0.3} + C⁴⁰⁰ + S^{0.1}; □, O^{0.3} + C⁴⁰⁰ + S¹.

plants (-37% of BE). At AE-0, the O_3 -inhibition of P_N in the $O^{0.1}+C^{400}+S^0$ plants showed slight amelioration by SA. That amelioration was clear at AE-3: P_N in the $O^{0.1}+C^{400}+S^{0.1}$, $O^{0.1}+C^{400}+S^1$, $O^{0.3}+C^{400}+S^{0.1}$, and $O^{0.3}+C^{400}+S^1$ plants were, respectively, 95%, 95%, 76% and 77% of those at BE (Fig. 2a). At AE-0, g_s in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{400}+S^0$ plants had decreased respectively to 40% and 23% of those at BE. Thereafter, they recovered somewhat. When recorded at AE-3, g_s of the corresponding plants were, respectively, 71% and 69% of those at BE. The g_s was ameliorated slightly by SA: g_s in the $O^{0.3}+C^{400}+S^1$ plants at AE-1 and the $O^{0.3}+C^{400}+S^1$ plants at AE-3 were, respectively, 76% and 77% of the $O^{0.3}+C^{400}+S^0$ plants (Fig. 2b). Three-way ANOVA for P_N and g_s showed clear effects of O_3 during AE-0 to AE-3 ($P \leq 0.001$, Table 1). It also showed no interaction between O_3 and SA because the effect of SA on O_3 -inhibition of P_N was extremely slight (Table 1).

As presented in Fig. 2c, F_v/F_m in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{400}+S^0$ plants decreased respectively to 92% and 77% of those at BE. Thereafter, the plants recovered from O_3 -inhibition. At AE-3, the $O^{0.1}+C^{400}+S^0$ plants had recovered fully, but the $O^{0.3}+C^{400}+S^0$ plants remained at 96% of BE. The O_3 -inhibition of F_v/F_m was unaffected by SA at AE-0 and AE-1, but at AE-3, the effect of $O^{0.1}$ on F_v/F_m was dispelled in the $O^{0.1}+C^{400}+S^{0.1}$ and $O^{0.1}+C^{400}+S^1$ plants (Fig. 2c). The F_q'/F_m' in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{400}+S^0$ plants decreased respectively to 79% and 63% of those at BE (Fig. 2d). Thereafter, the plants recovered from O_3 -inhibition. At AE-3, the $O^{0.1}+C^{400}+S^0$ plants recovered substantially (-11% of BE) but persisted in the $O^{0.3}+C^{400}+S^0$ plants (-17% of BE). At AE-1 and AE-3, the O_3 -inhibition of F_q'/F_m' in the corresponding plants was ameliorated slightly by SA application. At AE-3, the effect of $O^{0.1}$ on F_q'/F_m' disappeared in the $O^{0.1}+C^{400}+S^{0.1}$ and $O^{0.1}+C^{400}+S^1$ plants.

In addition, the F_q'/F_m' in the $O^{0.3}+C^{400}+S^{0.1}$ and $O^{0.3}+C^{400}+S^1$ plants at AE-3 were, respectively, 89% and 88% of those at BE. Three-way ANOVA for F_v/F_m and F_q'/F_m' showed clear negative effects of O_3 during AE-0 to AE-3 ($P \leq 0.001$, Table 1). It also showed no interaction between O_3 and SA because the effect of SA on O_3 -inhibition of F_v/F_m or F_q'/F_m' was extremely slight (Table 1).

Effect of SA on O_3 -inhibition of photosynthesis under elevated CO_2 (Exp. 1)

The O_3 -inhibition of all photosynthesis-related parameters was ameliorated by elevated CO_2 (Fig. 3). At AE-0, P_N in the $O^{0.1}+C^{800}+S^0$ and $O^{0.3}+C^{800}+S^0$ plants were, respectively, 89% and 75% of those at BE. Thereafter, P_N recovered fully in the $O^{0.1}+C^{800}+S^0$ plants at AE-1 and in the $O^{0.3}+C^{800}+S^0$ plants at AE-3. The effect of $O^{0.3}$ on P_N had disappeared in the $O^{0.3}+C^{800}+S^{0.1}$ and $O^{0.3}+C^{800}+S^1$ plants at AE-3 (Fig. 3a). At AE-0, g_s in the $O^{0.1}+C^{800}+S^0$ and $O^{0.3}+C^{800}+S^0$ plants had decreased respectively to 65% and 42% of those at BE. At AE-3, g_s in the $O^{0.1}+C^{800}+S^0$ plants had fully recovered but in the $O^{0.3}+C^{800}+S^0$ plants, it remained 79% of that at BE. The inhibition of O_3 on g_s in the $O^{0.1}+C^{800}+S^{0.1}$, $O^{0.1}+C^{800}+S^1$, $O^{0.3}+C^{800}+S^{0.1}$ and $O^{0.3}+C^{800}+S^1$ plants had disappeared at AE-3 (Fig. 3b). Three-way ANOVA for P_N and g_s showed clear interactions between O_3 and CO_2 from AE-0 to AE-3 ($P \leq 0.001$, Table 1).

The F_v/F_m in the $O^{0.3}+C^{800}+S^0$ plants at AE-0 decreased to 91% of that at BE. At AE-1, the $O^{0.3}+C^{800}+S^0$ plants fully recovered from O_3 -inhibition. However, it was not ameliorated by SA during AE-0 to AE-1 (Fig. 3c). The F_q'/F_m' in the $O^{0.3}+C^{800}+S^0$ plants at AE-0 was 75% of that at BE. Thereafter, they recovered substantially. The $O^{0.3}+C^{800}+S^0$ plants at AE-1 and AE-3 were, respectively, 83% and 89% of those at BE. The inhibition was ameliorated by SA.

Table 1 Statistical analysis of the effects of O_3 , CO_2 , and SA on net photosynthetic rate (P_N), stomatal conductance (g_s), maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II, and total (AA+DHA), reduced (AA), and oxidized (DHA) ascorbic acid contents per fresh weight in rice leaves shown in Figs. 2, 3, and 4 (Exp. 1). AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, and 1 d and 3 d after gas exposure. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, n.s. —not significant, by three-way ANOVA.

Time	Factor	P_N	g_s	F_v/F_m	F_q'/F_m'	AA+DHA	AA	DHA	RDS
AE-0	O_3	***	***	***	***	***	***	***	***
	CO_2	***	*	***	***	n.s.	n.s.	***	***
	SA	n.s.	n.s.	n.s.	n.s.	***	***	n.s.	*
	$O_3 \times CO_2$	***	***	***	***	n.s.	n.s.	*	*
	$O_3 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$O_3 \times CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AE-1	O_3	***	***	***	***	***	***	***	***
	CO_2	***	***	***	*	n.s.	*	***	***
	SA	*	*	n.s.	n.s.	***	***	n.s.	***
	$O_3 \times CO_2$	***	***	***	n.s.	n.s.	*	***	***
	$O_3 \times SA$	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	**
	$CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$O_3 \times CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AE-3	O_3	***	***	***	***	***	***	*	***
	CO_2	***	***	*	**	n.s.	*	*	***
	SA	***	n.s.	n.s.	n.s.	***	**	n.s.	n.s.
	$O_3 \times CO_2$	***	***	*	n.s.	n.s.	**	***	***
	$O_3 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$O_3 \times CO_2 \times SA$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

rated by SA at AE-1 and AE-3. At AE-1, F_q'/F_m' in the $O^{0.3}+C^{800}+S^{0.1}$ and $O^{0.3}+C^{800}+S^1$ plants were 87% of those at BE, and at AE-3, the effect of O₃ in the $O^{0.3}+C^{800}+S^{0.1}$ and $O^{0.3}+C^{800}+S^1$ plants had disappeared (Fig. 3d). Three-way ANOVA for F_v/F_m and F_q'/F_m' showed clear interaction between O₃ and CO₂ at AE-0 ($P \leq 0.001$, Table 1).

Effects of O₃ and SA on ascorbic acid content under different CO₂ concentrations (Exp. 1)

Because the effects of O₃ and SA on total ascorbic acid, AA and DHA contents expressed per fresh weight were similar to those expressed per leaf area, the values presented in Fig. 4 are those obtained on a fresh weight basis.

At AE-0, total ascorbic acid content in the $O^{0.3}+C^{400}+S^0$ plants decreased to 63% of that at BE but the O₃-inhibition was less under elevated CO₂ (C⁸⁰⁰). The content in the $O^{0.3}+C^{800}+S^0$ plants was 71% of that at BE. At AE-1, the content had decreased further. Contents in the $O^{0.1}+C^{400}+S^0$, $O^{0.3}+C^{400}+S^0$, and $O^{0.3}+C^{800}+S^0$ plants were, respectively, 83%, 55%, and 68% of those at BE. At AE-3, the total ascorbic acid content in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{800}+S^0$ plants had recovered fully but in the $O^{0.3}+C^{400}+S^0$ plants, the recovery remained low (−30% of BE). The O₃-inhibition of total ascorbic acid content was ameliorated by SA application. The amelioration became clearer with time (Fig. 4a, b).

At AE-0, RDS in the $O^{0.1}+C^{400}+S^0$, $O^{0.3}+C^{400}+S^0$, and $O^{0.3}+C^{800}+S^0$ plants decreased respectively to 88%, 80%, and 87% of those at BE. The O₃-inhibition of RDS was ameliorated by SA. At AE3, the plants fully recovered from O₃-inhibition in the $O^{0.1}+C^{400}+S^{0.1}$, $O^{0.1}+C^{400}+S^1$, $O^{0.3}+C^{800}+S^{0.1}$, and $O^{0.3}+C^{800}+S^1$ plants, but it persisted in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{800}+S^0$ plants. At AE-3, the plants fully recovered from O₃-inhibition in the $O^{0.1}+C^{400}+S^0$ and $O^{0.3}+C^{800}+S^0$, but it persisted in the $O^{0.3}+C^{400}+S^0$ (−80% of BE) and $O^{0.3}+C^{400}+S^{0.1}$ and $O^{0.3}+C^{400}+S^1$ (−

86% of BE) plants (Fig. 4c, d). Three-way ANOVA for total ascorbic acid and RDS showed clear negative effects of O₃ during AE-0 to AE-3 ($P \leq 0.001$, Table 1). In addition, because SA ameliorated the negative effect of O₃ on ascorbic acid content, three-way ANOVA for total ascorbic acid and RDS showed positive effects of SA from AE-0 to AE-3 (except RDS at AE-3) (Table 1).

Effects of O₃ and CO₂ on salicylic acid content and photosystem II (Exp. 2)

The trends of F_v/F_m and F_q'/F_m' resemble those in Exp. 1. At AE-0, F_v/F_m in the $O^{0.1}+C^{400}$, $O^{0.3}+C^{400}$ and $O^{0.3}+C^{800}$ plants had decreased respectively to 92%, 85% and 88% of the values recorded at BE; the F_q'/F_m' in the corresponding plants had decreased respectively to 85%, 71%, and 80% of those at BE. Thereafter, the F_v/F_m and F_q'/F_m' recovered from O₃-inhibition; at AE-3, the $O^{0.1}+C^{400}$ and $O^{0.3}+C^{800}$ plants had recovered fully, but in the $O^{0.3}+C^{400}$ plants the inhibition remained, respectively, at 97% and 84% of those recorded at BE (Fig. 5a, b).

The SA contents in the O^0+C^{400} and O^0+C^{800} plants were 11–15 $\mu\text{g g}^{-1}$ FW. These are within a range of rice leaves (8–30 $\mu\text{g SA g}^{-1}$ FW) reported by Silverman et al. (1995). The SA content in the $O^{0.3}+C^{400}$ plants at AE-0 increased to 160% of that at BE. At AE-1, it increased further: in the $O^{0.3}+C^{400}$ and $O^{0.3}+C^{800}$ plants, the contents were, respectively, 252% and 164% of those at BE. At AE-3, the SA contents in the $O^{0.1}+C^{400}$ and $O^{0.3}+C^{800}$ plants increased further, but that in the $O^{0.3}+C^{400}$ plant was lower than that at AE-1 (Fig. 5c). Two-way ANOVA for SA content showed a clear effect of O₃ from AE-1 to AE-3 ($P \leq 0.001$, Table 2).

DISCUSSION

Coincident with our previous results reported for paddy rice (Imai and Kobori, 2008; Kobayakawa and Imai,

Fig. 3 Effects of O₃ and SA on net photosynthetic rate (P_n), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II at elevated CO₂ concentration in rice leaves (Exp. 1): vertical bars represent LSD ($P \leq 0.05$); BE, AE-0, AE-1, AE-3 — before, immediately after, 1 d and 3 d after gas exposure; O^0 , $O^{0.1}$, $O^{0.3}$ — 0, 0.1, 0.3 $\text{cm}^3 \text{m}^{-3}$ O₃; C^{400} , C^{800} — 400, 800 $\text{cm}^3 \text{m}^{-3}$ CO₂; S^0 , $S^{0.1}$, S^1 — 0, 0.1, 1 mM SA. ● — $O^0+C^{800}+S^0$; ○ — $O^0+C^{800}+S^{0.1}$; △ — $O^0+C^{800}+S^1$; ▲ — $O^{0.1}+C^{800}+S^0$; ▲ — $O^{0.1}+C^{800}+S^{0.1}$; △ — $O^{0.1}+C^{800}+S^1$; ■ — $O^{0.3}+C^{800}+S^0$; ■ — $O^{0.3}+C^{800}+S^{0.1}$; □ — $O^{0.3}+C^{800}+S^1$.

Fig. 4 Effects of O_3 and SA on total ascorbic acid and redox state of ascorbic acid (RDS) at ambient (a, b) and elevated (c, d) CO_2 concentrations in rice leaves (Exp. 1): vertical bars represent LSD ($P \leq 0.05$); AA, reduced form of ascorbic acid; DHA, dehydroascorbic acid (oxidized form of ascorbic acid); RDS, redox state of ascorbic acid [AA/(AA+DHA)]; BE, AE-0, AE-1, AE-3 — before, immediately after, 1 d and 3 d after gas exposure; O^0 , $O^{0.1}$, $O^{0.3}$ — 0, 0.1, 0.3 cm³ m⁻³ O_3 ; C^{400} , C^{800} — 400, 800 cm³ m⁻³ CO_2 ; S^0 , $S^{0.1}$, S^1 — 0, 0.1, 1 mM SA; ●, $O^0 + C^{400}$ or $C^{800} + S^0$; ●, $O^0 + C^{400}$ or $C^{800} + S^{0.1}$; ○, $O^0 + C^{400}$ or $C^{800} + S^1$; ▲, $O^{0.1} + C^{400}$ or $C^{800} + S^0$; ▲, $O^{0.1} + C^{400}$ or $C^{800} + S^{0.1}$; △, $O^{0.1} + C^{400}$ or $C^{800} + S^1$; ■, $O^{0.3} + C^{400}$ or $C^{800} + S^0$; ■, $O^{0.3} + C^{400}$ or $C^{800} + S^{0.1}$; □, $O^{0.3} + C^{400}$ or $C^{800} + S^1$.

Fig. 5 Effects of O_3 and CO_2 on maximum (F_v/F_m), operating (F_q'/F_m') quantum efficiency of photosystem II (a, b) and salicylic acid (SA) contents in rice leaves (Exp. 2): vertical bars represent LSD ($P \leq 0.05$); BE, AE-0, AE-1, AE-3 — before, immediately after, 1 d and 3 d after gas exposure; O^0 , $O^{0.1}$, $O^{0.3}$ — 0, 0.1, 0.3 cm³ m⁻³ O_3 ; C^{400} , C^{800} — 400, 800 cm³ m⁻³ CO_2 ; ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

2011a), the photosynthesis-related parameters, and ascorbic acid content and its RDS were degraded by O_3 exposure. They were ameliorated by elevated CO_2 (C^{800}) (Figs. 2–5). Generally, the amelioration of O_3 -induced damage of plants by elevated CO_2 is largely attributed to the restriction of CO_2 intake through stomatal closure (Booker and Fiscus, 2005; Imai and Kobori, 2008), although undetermined additional factors may be concerned. In the present study, SA application slightly ameliorated the O_3 inhibition of P_N and PSII (F_v/F_m and F_q'/F_m'), as in the case of MeJA application (Kobayakawa and Imai, 2012b), but the amelioration by SA was less than that of MeJA. In addition, photosynthesis-related parameters were unaffected by SA (Figs. 2 and 3).

In contrast to SA, MeJA decreased gas-exchange-related parameters (Popova et al., 1988; Kobayakawa and Imai, 2012b). Kobayakawa and Imai (2012b) showed that MeJA application ameliorated the O_3 inhibition of P_N in rice leaves through closing stomata and promoting antioxidant capacity, whereas MeJA decreased P_N through closing stomata in healthy leaves (O^0 plants). The SA did not affect g_s in this study, although one report in the literature has described SA-induced stomatal closure (Rivas-San Vicente and Plasencia, 2011). Therefore, because the O_3 flux through stomata did not decrease by SA, the O_3 -inhibition of P_N at AE-0 was not entirely ameliorated by SA.

The SA and SA derivatives induce systemic acquired

Table 2 Statistical analysis of the effects of O₃ and CO₂ on maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II, and salicylic acid content per fresh weight in rice leaves presented in Fig. 5 (Exp. 2). AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, and 1 d and 3 d after gas exposure. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, n.s. — not significant, by two-way ANOVA.

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

resistance (Oostendorp et al., 2001). Similarly, SA application ameliorates various stresses caused by factors such as heavy metals, salinity, and temperature (Hayat et al., 2010). Zhao et al. (2010) evaluated the effects of exogenously applied SA (0.5–1 mM) on chronic O₃ stress in soybean. They showed that O₃-induced decreases in superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities, ascorbic acid and glutathione contents were ameliorated by SA, but its ability to ameliorate injury by O₃ exposure was limited. In *Arabidopsis*, the O₃ injury was ameliorated by SA through the promotion of glutathione biosynthesis (Yoshida et al., 2009a). Furthermore, in wheat, exogenously applied SA promoted ascorbate peroxidase (APX) and glutathione reductase (GR) activities (Agrawal et al., 2005). In the present study, the O₃-induced inhibition of P_N was ameliorated slightly by SA application (Figs. 2 and 3). In addition, the decreases in total ascorbic acid and RDS induced by O₃ were ameliorated by SA application, although the total ascorbic acid was unaffected by SA in healthy leaves of the O⁰ plants (Fig. 4). Presumably, SA accelerates the recovery of P_N from O₃ inhibition because SA maintains homeostasis of ascorbic acid through increases in glutathione content and ascorbic acid-glutathione cycle-related enzyme activities rather than the promotion of ascorbic acid biosynthesis. The SA might be used as an agrochemical under field conditions to alleviate stresses because no P_N depletion is found by SA application, unlike MeJA application (Popova et al., 1988; Kobayakawa and Imai, 2012b).

Coincident with the reports on *Arabidopsis* (Sharma et al., 1996; Rao et al., 2000), tobacco (Yalpani et al., 1994), and poplar (Koch et al., 2000), the O₃ exposure increased the endogenous level of SA in rice leaves (Fig. 5c). Furthermore, SA content was increased by salt stress in rice leaves (Sawada et al., 2006). In the defense response of plants, SA has a function promoting cell-death during O₃ injury (Kangasjärvi et al., 2005). The present study showed that SA content was greater by O₃ at AE-1 and AE-3 than at AE-0. Therefore, SA may be related to the enlargement of O₃ injury or induction of recovery process, or both. Ogawa et al. (2005) assumed that low concentrations of SA induced the defense responses, but its high concentrations induced cell death. The SA contents of rice leaves (8–30 $\mu\text{g g}^{-1}$ FW) are higher than that of *Arabidopsis*

(0.01–0.1 $\mu\text{g g}^{-1}$ FW) (Ogawa et al., 2006). Consequently, threshold doses of SA in the mechanism of defense induction and cell death might differ among plant species. The present study showed that the effect of SA application is not concentration-dependent (Figs. 2–4). Therefore we infer that SA does not act as a direct defense component, but instead acts as a signal for inducing defense response in O₃-exposed leaves.

DeLucia et al. (2012) reported that elevated CO₂ stimulated the production of endogenous SA in plants. However, in the present experiment, SA contents were unaffected by the CO₂ concentration (C^{400} vs. C^{800}) in rice leaves. Moreover, DeLucia et al. (2012) showed that elevated CO₂ suppressed the production of endogenous JA. Kobayakawa and Imai (2012b) found that the effect of MeJA application on O₃-inhibition was suppressed slightly by elevated CO₂ (C^{800}). In *Arabidopsis*, syntheses of plant hormones such as indoleacetic acid, gibberellin, and cytokinin are promoted, but abscisic acid is suppressed by elevated CO₂ (Teng et al., 2006). These changes might alter the physiological responses of plants including the defense response and sensitivity to various stresses. For example, rice plants grown under elevated CO₂ are more susceptible to leaf blast than those grown under ambient CO₂ (Kobayashi et al., 2006). In addition, elevated CO₂-grown soybean plants show a higher sensitivity to acute exposures of O₃ because of the lowered antioxidant capacity under elevated CO₂ (Gillespie et al., 2011).

Actually, SA, JA, and ethylene mutually interact and form a complex network (Rao and Davis, 2001; Kangasjärvi et al., 2005). Rao and Davis (2001) proposed a model in which the SA induces not only defense gene expression through the activation of MAP kinases, but also ethylene biosynthesis. Therefore, to clarify the relations between O₃ responses of paddy rice and plant hormones under elevated CO₂ concentrations, additional studies must be conducted, including studies of key compounds such as jasmonates and ethylene.

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