大気中オゾンおよび二酸化炭素の濃度に対する水稲の生理的反応に関する研究

Dissertation

Studies on the Physiological Responses of Paddy Rice to Atmospheric Concentrations of Ozone and Carbon Dioxide

（大気中オゾンおよび二酸化炭素の濃度に対する水稲の生理的反応に関する研究）

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Contents

Abbreviations

AA: reduced form of ascorbic acid

ACC: 1-aminocyclopropanne-1-carboxylic acid

APX: ascorbate peroxidase

AVG: aminoethoxyvinylglycine

CAT: catalase

C_i: intercellular CO_2 concentration

CO_2: carbon dioxide

DHA: dehydroascorbic acid

DHAR: dehydroascorbate reductase

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

F': steady fluorescence

F_0: minimum fluorescence in the dark-adapted state

F_0': minimum fluorescence in the steady state

Fd: ferredoxin

F_m: maximum fluorescence in the dark-adapted state

F_m': maximum fluorescence in the steady state

F_q': difference between F_m' and F'

F_q'/F_m': operating quantum efficiency of PSII

F_v: variable fluorescence

F_v': variable fluorescence in the steady state

F_v/F_m: maximum quantum efficiency of PSII

F_v'/F_m': maximum quantum efficiency at the given PPFD

FW: fresh weight

GOGAT: glutamate synthase

GR: glutathione reductase

g_s: stomatal conductance

GSH: glutathione

GSSG: oxidized glutathione

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

JA: jasmonic acid

JMT: jasmonic acid carboxyl methyltransferase

MA: (+)-mandelic acid

MeJA: methyl jasmonate

NiR: nitrite reductase

NR: nitrate reductase

O_3: ozone

PAL: phenylalanine ammonia-lyase

PCD: programmed cell death

PMSF: phenylmethylsulfonyl fluoride

P_N: net photosynthetic rate

POD: peroxidase

PPFD: photosynthetic photon flux density

PSII: photosystem II

PVP: polyvinylpyrrolidone

Q_A: primary quinone acceptor of PSII

q_E: energy (ΔpH)-dependent quenching coefficient

q_I: photoinhibitory quenching coefficient

q_N: non-photochemical quenching coefficient

qp: photochemical quenching coefficient

q_T: state-transition quenching coefficient

R_D: respiration rate

RDS: redox state

ROS: reactive oxygen species

Rubisco: ribulose 1,5-bisphosphate carboxylase/oxygenase

SA: salicylic acid

SAM: S-adenosyl-methionine

SiR: sulfite reductase

SLW: specific leaf weight

SOD: superoxide dismutase

STS: silver thiosulfate complex

VPD: vapor pressure deficit

Chapter 1

Introduction

1 – 1. Air pollutants and plant responses

Air pollutants such as photochemical oxidants, sulfur dioxide (SO_2), nitrogen oxides (NO_2, NO), and PM2.5 are well known to be toxic to humans and plants. In the summer of 1970, 'photochemical smog' was reported for the first time in Japan (Nouchi, 2001). Photochemical oxidants are generated by complex photochemical reactions involving ultraviolet rays and nitrogen oxides and hydrocarbons emitted from automobiles and factories under warm, sunny, and windless weather. The Japanese environmental quality standard for photochemical oxidants is set as < 0.06 cm^3 m^{-3}; when it exceeds 0.12 and 0.24 cm^3 m^{-3}, local public bodies must, under the regulations, respectively issue an oxidant warning and an alarm (Nakanishi et al., 2009). Of photochemical oxidant components, 90% or more is ozone (O_3) (Cabrera et al., 1988; Nouchi, 2001). Stratospheric O_3 protects Earth from ultraviolet rays, but tropospheric O_3 poses a health hazard to humans. It attacks all plant species and decreases their photosynthesis capabilities and growth rates concurrently with white-dot symptoms on the leaves of herbaceous species and reddish-to-black-dot symptoms on the leaves of arboreous, leguminous, and gramineous species. Within cells, it damages organelles and biopolymers, including DNA (Nouchi, 2001; Roshchina and Roshchina, 2003). Anthropogenic emissions of chemical reactive gases have caused tropospheric O_3 increases, particular in the northern Hemisphere. In Europe, the upward O_3 trend seems most alarming: 5–20% per decade (Guicherit and Roemer, 2000). There is also growing concern that the long-distance transport of air pollution increases the background O_3 concentration (Ohara, 2011).

In the Kanto region of Japan, where rice is cultivated as a staple summer crop, 10–20

warnings are received every growing season. Hourly peak values are sometimes close to 0.2 cm^3 m^{-3} (Environ. Improve. Div. Tokyo Metro. Bureau Environ., 2005). Kobayashi (1999) estimated that O_3 decreased rice production by up to 10% in the Kanto region of Japan during 1981–1985. This deterioration was ascribed to photosynthesis-related processes rather than fertilization at flowering (Imai and Ookoshi, 2011), as was the case for winter wheat (Feng et al., 2007). Consequently, it is necessary to elucidate the effects of acute (single or repeated) O_3 exposure on rice plant physiology, such as photosynthesis, as a basis for reductions in dry-matter production and yield formation of this crop in fields where acute photochemical oxidants appear, along with chronic exposure.

When exposed to O_3, rice plants suffer damage: inhibition of net photosynthetic rate (P_N) and stomatal conductance (g_s) (Imai and Kobori, 2008), decreased ribulose 1, 5-bisphosphate carboxylase/oxygenase (Ishioh and Imai, 2005), chlorophyll and carotenoid contents (Rai and Agrawal, 2008), in addition to visible leaf-related symptoms (Imai and Kobori, 2008) and breakdown of the cellular ultrastructure (Toyama et al., 1989). Moreover, O_3 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). Detrimental effects of O_3 have also been reported in many crops and trees (Ainsworth et al., 2012; Bhatia et al., 2012).

To maintain rice yield, it is necessary to ascertain the role of O_3 in both carbon fixation and photochemical reactions. Imai and Kobori (2008) examined the P_N, g_s, and P_N/C_i-curve and concluded that the O_3-induced decline in P_N was caused largely by decreased g_s.They also found that high concentrations of O_3 caused damage to photochemical mechanisms and/or carbon reduction systems of chloroplast as well as damage to stomatal mechanisms. However, this was not the sole reason. Other components of photosynthetic process, such as the photosystem, were affected by O_3. Fortunately, the use of chlorophyll fluorescence measurements has become an established practice to diagnose changes in photosystem II (PSII) attributable to environmental stresses such as excessive light and water stress. Numerous studies of the effects of environmental

stress on PSII have been conducted (Adams and Demming-Adams, 2004). However, few reports describe the effect of O_3 on the photochemical reaction of photosynthesis in rice leaves, e.g., PSII is adversely affected by chronic exposure (Rai and Agrawal, 2008, Pang et al., 2009).

1 –2. Interactive effects of O_3 and CO_2, and related antioxidants and plant hormones

Concurrent with O_3 air pollution, the global atmospheric CO_2 concentration has increased from the pre-industrial value of about 280 cm^3 m^{-3} to the current level of 391 cm^3 m^{-3}. This trend is expected to continue unless human activities are curtailed substantially (WMO WDCGG, 2013). Elevated CO_2 induces various responses such as the promotion of photosynthesis, stomatal closure, increased leaf thickness and yield, and changes of metabolism including plant hormone and antioxidants (Imai, 1988; Gillespie et al., 2011; Kirkham, 2011; DeLucia et al., 2012). Because elevated CO_2 concentrations decrease g_s, they ameliorate O_3-induced injury by suppressing O_3 intake through the stomata (Booker and Fiscus, 2005). Donnelly et al. (2000) showed that chronic O_3 (ambient plus 50 cm^3 m^{-3})-induced adverse effects on the photosynthetic activity and chlorophyll contents were ameliorated by elevated CO_2 (680 or 510 cm^3 m^{-3}) in a spring wheat. By exposing rice leaves to combinations of O_3 and CO_2, Ishioh and Imai (2005) found that elevated CO_2 ameliorated the decline in P_N, Rubisco and chlorophyll caused by O_3.

Although production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) is low under normal conditions, it has been emphasized that disruption the cellular homeostasis of cells enhances ROS production. Such stresses include drought stress, salt stress, heat shock, high light stress, heavy metals, ultraviolet radiation, and air pollutants such as O_3 and SO_2. Because ROS induces cell death, the higher plants have antioxidant metabolism to scavenge excess ROS. Antioxidant metabolism is divided into two groups: enzymatic and non-enzymatic. The former is divisible into water-soluble non-enzymatic antioxidants such as ascorbic acid and glutathione, and lipid-soluble antioxidants such as α-tocopherol and xanthophyll. The latter includes superoxide dismutase

(SOD), peroxidase (POD), and catalase (CAT) (Allan and Fluhr, 2007; Mühlenbock et al., 2007; Taiz and Zeiger, 2010). These antioxidants perform not only ROS degradation but also other functions. For example, glutathione has a vital role in biosynthesis of phytochelatin (Ogawa, 2002), and regulation of photosynthesis-related enzymes and stomatal closure (Ito et al., 2003; Jahan et al., 2008). Therefore, the change of ROS and antioxidants level causes various physiological responses in plants. Ozone is known to promote ROS production. Therefore, antioxidant metabolism is an important determination factor for O_3 injury (Sharma and Davis, 1997).

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 O_3 stress (Kangasjärvi et al., 2005; Rao and Davis, 2001) in studies using various mutants and molecular biology techniques. In addition, these hormone contents are increased by O_3 in *Arabidopsis*, tobacco and popular (Rao and Davis, 2001; Tamaoki, 2008). The complex network including these hormones (JA, SA and ethylene), ROS signaling and MAP kinase cascade are inferred to exist in plants (Rao and Davis, 2001). However, these mechanisms have been detected only in model plants, except that ethylene production from rice leaves has been increased by O_3 (Ohki et al., 1999).

1 – 3. Study objectives

Given the background described above, the author conducted experiments of several types to clarify the effects of O_3 and CO_2 as a basis of paddy rice cultivation for the future atmospheric conditions. Because photosynthesis is an important process determining rice production, effects on net photosynthetic rate and photosystem II activity were measured as indicators of these two gases. In addition, this study examined the role of stress response-related metabolism such as antioxidant and plant hormones to elucidate their relations to rice plant growth and development.

In Chapter 2, the author establishes the optimum dark adaptation period for evaluating F_v/F_m in O_3-exposed rice leaves as a key technique to analyze PSII activity in rice leaves. The results of

Chapter 2 are used for the estimation of F_v/F_m after Chapter 3. In Chapter 3, the author estimates the effects of O_3 and CO_2 on photosynthesis including carbon fixation reaction and photosystem using gas-exchange and chlorophyll fluorescence measurements. In addition, the author compares the effects at different growth stage (vegetative growth stage vs. heading stage). In Chapter 3 Sections 2 and 3, the author discusses the determining factor for O_3-inhibition of photosynthesis, i.e., antioxidant level and leaf inclination angle. In Chapter 3 Section 4, the responses of rice to the exposure pattern of elevated CO_2 were examined: acute (only one day exposure) or chronic (from sowing to day of exposure). In that section, effects of acute O_3 exposure on photosynthesis under different growth CO_2 concentrations are examined because combinations of acute O_3 and chronic elevated CO_2 are predicted for future atmospheric conditions. In Chapter 4, the author examines effects of O_3 and CO_2 on nitrate reduction because nutrient nitrogen is an important factor for plant growth including photosynthesis activity. Chapter 5 describes the effects of plant hormones on O_3-inhibition of photosynthesis in rice leaves. Chapter 5 Sections 1–3 respectively explain the relation between O_3-inhibition of photosynthesis and jasmonic acid, salicylic acid and ethylene in rice grown under different CO_2 concentrations. All sections demonstrate the effects of exogenous plant hormones and quantifications of endogenous plant hormone in rice leaves. In Chapter 6, the author discusses physiological responses of rice to atmospheric concentrations of O_3 and CO_2 in this study, in comparision to other related research works. Finally, the author puts forward concluding remarks including contribution to future rice cultivation.

Chapter 2

Optimum Dark Adaptation Period for Evaluating the Maximum Quantum Efficiency of Photosystem II in the O_3-Exposed Leaves of Paddy Rice

2 – 1. Introduction

Among the indicators obtained through performing chlorophyll fluorescence measurements, maximum quantum efficiency of PSII (F_v/F_m) is used most frequently. F_v/F_m is determined from the easiest and simplest type of chlorophyll fluorescence measurement. However, before such measurements can be obtained, knowledge regarding the dark adaptation period is required. Generally, the dark adaptation period for the leaves subjected to this treatment is 30 min. However, Sonoike (2009) observed that if the dark adaptation period is prolonged, the effects of short-term stress on PSII will disappear. In fact, many researchers (Lee, 1991; Guidi et al., 1997, 2000a, b; Shavnin et al., 1999; Calatayud et al., 2002; Degl'Innocenti et al., 2002; Skotnica et al., 2003; Flowers et al., 2007; Wang et al., 2009) have measured the effect of O_3 on F_v/F_m based on qualification of chlorophyll fluorescence under various dark adaptation periods, the shortest of which was 5 min (Skotnica et al., 2003), and the longest was 60 min (Guidi et al., 2000b; Flowers et al., 2007).

This chapter described the establishment of optimum dark adaptation period for the measurement of F_v/F_m in rice leaves under O_3 stress or stress free conditions to clarify the inhibition and recovery of photosynthesis by O_3.

2 – 2. Mechanism of chlorophyll fluorescence

Light energy absorbed by chlorophylls associated with PSII can be used to drive photochemistry in which an electron is transferred from the reaction center chlorophyll, P680, to the primary quinone (Q_A) acceptor of PSII. Alternatively, absorbed light energy can be lost from PSII as

chlorophyll fluorescence or heat. If the rate of one process increases, the rates of the other two will decrease (Fig. 2–1). Fig. 2–2 shows the change of chlorophyll fluorescence during measurement. The measurement of chlorophyll fluorescence is separated two steps, namely (i) dark-adapted state (non-steady state) and (ii) light-adapted state (steady state). When a leaf is kept in the dark, the Q_A becomes maximally oxidized. The PSII reaction centers can then perform photochemical reduction of Q_A, and the minimal fluorescence in the dark-adapted state (F_0) can be determined. Thereafter, when a leaf is exposed to a short saturating light pulse of high PPFD (typically of less than 1 s at several thousand μmol m^{-2} s^{-1}), Q_A reaches a maximally reduced state, and the maximal fluorescence in the dark-adapted state (F_m) can be determined. Baker (2008) described variable fluorescence (F_v) as the difference between F_0 and F_m values. Generally, the dark adaptation period for the leaves subjected to this treatment is 30 min. A leaf in continuous actinic light (steady state) has a fluorescence level termed F', which rises to the maximal fluorescence level, F_m', when the leaf is exposed to a saturating light pulse that maximally reduces Q_A. The difference between F_m' and F' is F_q'. Then, after removing the actinic light to maximally oxidize Q_A, F_0' is measured by exposing the leaf to pulse of weak far-red light. The maximum quantum efficiency (F_v/F_m), operating efficiency (F_q'/F_m'), maximum efficiency at the given PPFD (F_v'/F_m') and the photochemical (q_p) and non-photochemical (q_N) quenching coefficients of PSII were obtained using the following equations (Baker, 2008, Sonoike, 2009):

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

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

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

$$q_p = (F_m' - F')/(F_m' - F_0')$$

$$q_N = 1 - (F_m' - F_0')/(F_m - F_0)$$

Table 2–1 shows the definition and physiological relevance of chlorophyll fluorescence parameters used in this study (Baker, 2008).

2 – 3. Materials and methods

Plant materials and gas exposure

Two independent experiments were conducted in April and July and were designated Exp. 1 (April) and Exp. 2 (July). Japonica rice (*Oryza sativa* L. cv. Koshihikari) seeds were sown directly in Wagner pots (1/5000a) filled with dry soil (ca. 2.2–2.5 kg) and 12.5 g of compound fertilizer (N, P_2O_5, K_2O = 8, 8, 8%). The plants were grown in natural-light gas-exposure 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) under 70% RH and 400 cm^3 m^{-3} CO_2. Immediately after full expansion of the 8th leaves (Haun index = 8.0; Haun, 1973), the plants were exposed to 0, 0.1, or 0.3 cm^3 m^{-3} O_3 (expressed as O^0, $O^{0.1}$, or $O^{0.3}$, respectively) for 5 h during the day (08:00–13:00 local time). O_3 was supplied using a high voltage ozone generator with dry air (ED-OG-R6; Ecodesign Inc., Ogawa, Saitama, Japan). CO_2 was supplied from cylinders containing liquid CO_2. These gases were injected into air that had been charcoal-filtered. The O_3 and CO_2 concentrations were measured and computer controlled using an ultraviolet absorption-type O_3 analyzer (EG-2001F; Ebara Jitsugyo, Tokyo, Japan) and an infrared CO_2 analyzer (ZRH, Fuji Electric Systems, Tokyo, Japan), respectively.

Chlorophyll fluorescence measurements

A fluorometer (LI-6400-40; Li-Cor Inc., Lincoln, NE, USA) attached to a portable photosynthesis and transpiration measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA) and a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany) were used to measure the chlorophyll fluorescence of the 8th leaves from 0.1–1.1 h after O_3 exposure for each of five replicate plants. Chlorophyll fluorescence parameters were determined in these plants by applying 0.2 and 7,000 μmol m^{-2} s^{-1} of measuring light and a saturating pulse (0.8 s). Prior to the fluorescence measurements, the leaves were kept in the dark for 0, 1, 5, 10, 20, or 30 min (expressed as D^0, D^1, D^5, D^{10}, D^{20}, and D^{30}, respectively). Subsequently, minimum fluorescence

(F_0) and maximum fluorescence (F_m) were determined by irradiating the measuring light and saturating pulses, respectively. In addition to F_v/F_m, $(1/F_0) - (1/F_m)$ was calculated as an indicator of PSII photoinactivation (Havaux et al., 1991; Baker, 2008; Sonoike, 2009). The dark adaptation treatments and fluorescence measurements were conducted at 28 °C. In Exp. 2, chlorophyll fluorescence was measured using only a portable fluorometer (MINI-PAM; Heinz Walz GmbH, Effeltrich, Germany).

Statistical analysis

All chlorophyll fluorescence-related data were subjected to a two-way analysis of variance (ANOVA). The data were further subjected to a multiple comparison by Tukey's test to clarify the effects of O_3 concentrations with elapsing dark adaptation period. Statistical analyses were performed using Excel Statistics 2010 for Windows software package (Social Survey Research Information Co. Ltd., Tokyo, Japan). Differences among treated samples were considered statistically significant at $P \leq 0.05$ or $P \leq 0.01$ compared with non-treated plant group at each time interval. Appropriate standard errors of the means (SE) were calculated, and the results are presented as line graphs.

2 – 4. Results

In Exp. 1, the F_0, F_m and F_v/F_m values measured using one fluorometer (MINI-PAM) were slightly lower than those measured using the other fluorometer (LI-6400-40); however, the trends were similar (Fig. 2–3). Thus, this chapter employed MINI-PAM fluorometer in the replicate experiment (Exp. 2) because it was easier to carry than the LI-6400-40 fluorometer. The F_v/F_m ratio was lowest at D^0 in all treatments, and F_v/F_m was decreased in an O_3-concentration-dependent manner. In the O^0 plants, F_v/F_m increased from D^0 to D^{10} and was then nearly constant from D^{10} to D^{30}. The F_v/F_m ratio in the $O^{0.1}$ plants increased from D^0 to D^{10}, as in the O^0 plants, but then increased further after D^{10}. In the $O^{0.3}$ plants, F_v/F_m increased from D^0 to

D^5 and then tended to decrease with the time in darkness (Fig. 2–3A, B). F_0, a component of F_v/F_m, decreased slightly with the period of darkness in the O^0 plants. In contrast, in the $O^{0.3}$ plants, F_0 increased with the period of darkness: the F_0 measured at D^{30} had increased to 135% (MINI-PAM) – 154% (LI-6400-40) of its value at D^0 (Fig. 2–3C, D). F_m, a component of F_v/F_m, was decreased by $O^{0.3}$ though it substantially increased with the period of darkness (Fig. 2–3E, F). $(1/F_0) - (1/F_m)$, which is an indicator of PSII inactivation, increased in the O^0 plants, was unchanged in the $O^{0.1}$ plants, and decreased in the $O^{0.3}$ plants with the period of darkness (Fig. 2–3G, H). Statistical analyses (Tables 2–2 and 3) examining the O_3 inhibition of F_v/F_m, F_0, F_m, and $(1/F_0) - (1/F_m)$ support the description provided above regarding the fluorometers (LI-6400-40 and MINI-PAM), with special reference to the dark adaptation period.

In Exp. 2, F_v/F_m was found to be decreased by O_3 treatment, while F_0 was increased (Fig. 2–4A, B), as observed in Exp. 1. However, the trend found for F_m differed from that observed in Exp. 1. The value of F_m recovered dramatically with the period of darkness; however, because the decrease that occurred at D^0 was more pronounced than in Exp. 1, this recovery was insufficient (Fig. 2–4C). $(1/F_0) - (1/F_m)$ was decreased by O_3 treatment, as in Exp. 1, and this value decreased gradually with the period of darkness in the $O^{0.3}$ plants (Fig. 2–4D).

Statistical analyses (Tables 2–2 and 3) of the results obtained using a single fluorometer (MINI-PAM) supported the existence of differential responses to early dark adaptation periods in the two experiments. In Exp. 2, F_0, F_m, and $(1/F_0) - (1/F_m)$ showed lower significant levels than those of Exp. 1 when compared to each of the control plants (O^0) at early dark adaptation periods.

2 – 5. Discussion

In this chapter, F_v/F_m was adversely affected by O_3. The F_v/F_m ratios in all plants were lowest at D^0. In the O^0 and $O^{0.1}$ plants, F_v/F_m recovered with the period of darkness; however, because F_v/F_m decreased gradually with the period of darkness in the $O^{0.3}$ plants, the results of this chapter inferred that the inhibition of PSII by O_3 was exacerbated (Figs. 2–3A, B and 4A). F_v/F_m

decreases when F_0 increases and/or F_m decreases (i.e., $F_v = F_m - F_0$) (Baker, 2008; Sonoike, 2009). In this chapter, the F_0 values recorded in the $O^{0.1}$ and $O^{0.3}$ plants were generally higher than in the O^0 plants at all measurement times, and the values increased gradually with the progression of dark adaptation (Figs. 2–3C, D and 4B). Previous studies in snap bean (Lee, 1991; Flowers et al., 2007) and *Betula pendula* (Shavnin et al., 1999) support the current results that F_0 was increased by O_3 exposure. The increase in F_0 and decrease in F_m are induced by the inhibition of electron transport from Q_A to Q_B and the oxygen-evolving system from the manganese cluster to the tyrosine residue of the D1 protein, respectively (Sonoike, 2009). Therefore, it appeared that the damage that occurred to PSII in the $O^{0.3}$ plants was induced mainly by the inhibition of electron transport from Q_A to Q_B. In addition, as $(1/F_0) - (1/F_m)$ was found to be decreased in the $O^{0.3}$ plants with the period of darkness (Figs. 2–3G, H and 4D), PSII was gradually inactivated even during dark adaptation. However, F_m was observed to be decreased by O_3 treatment at any measurement time in Exp. 2. The F_v/F_m obtained at D^0 in Exp. 2 was lower than in Exp. 1. As the O_3-induced decrease in F_m observed in Exp. 2 (Fig. 2–4C) was more pronounced than that in Exp. 1 (Fig. 2–3E, F), it is possible that the detrimental effect of O_3 was more pronounced in Exp. 2 due to the higher light intensities involved. In fact, the PPFD values recorded at 12:00 in the natural light growth chamber were approximately 900 and 1,100 μmol m^{-2} s^{-1} in Exp. 1 and Exp. 2, respectively. Guidi et al. (2000b) also observed that the inhibition of P_N and PSII activity by O_3 was greater under high light intensities (30–1,000 μmol m^{-2} s^{-1} PPFD). While the decrease in F_v/F_m was induced by an increase in F_0 in the present study, in previous studies, a decrease in F_v/F_m was found to be induced by a decrease in F_m in O_3-exposed lettuce (Calatayud et al., 2002) and tobacco (Degl'Innocenti et al., 2002). Guidi et al. (2000a) measured the effect of O_3 on chlorophyll fluorescence in 14 bean cultivars and reported that the cause of the inhibition of F_v/F_m (e.g., increased F_0 and/or decreased F_m) depended on the cultivar. Interestingly, the F_v/F_m was decreased by both an increase in F_0 and a decrease in F_m in chronically O_3-exposed rice leaves (Rai and Agrawal, 2008). Therefore, the cause of the decrease in F_v/F_m depends on the species,

cultivar, and environmental conditions involved.

F_v/F_m is determined without irradiating actinic light. Therefore, the obtained values can only be decreased by the non-photochemical quenching coefficient (q_N). Three major components of q_N have been identified in plant leaves *in vivo*: the energy (ΔpH)-dependent quenching coefficient (q_E), photoinhibitory quenching coefficient (q_I), and state-transition quenching coefficient (q_T). The relaxation kinetics of these components is different: the half time ($t_{1/2}$) is 1 min for q_E, 5–10 min for q_T, and >30 min for q_I (Adams III and Demming-Adams, 2004; Krause and Jahns, 2004). Because q_T is suppressed under strong light, it should not be regarded as a photoprotective mechanism. In this chapter, the leaves of the plants were exposed to O_3 during sunny daytime hours. Therefore, F_v/F_m was decreased mainly by q_E and q_I. q_I includes the xanthophyll cycle-dependent component and inactivation of the D1 protein component. The relaxation time of these two components differs: the $t_{1/2}$ of the former is shorter than 30 min, whereas that of the latter is longer than 1 h (Adams III and Demming-Adams, 2004; Krause and Jahns, 2004). In all plants, F_v/F_m recovered dramatically from D^0 to D^1 (Figs. 2–3A, B and 4A). This recovery was likely induced by the relaxation of q_E. Consequently, in addition to the $O^{0.1}$ and $O^{0.3}$ plants, a decrease in F_v/F_m caused by q_E also occurred in the O^0 (control) plants. Furthermore, as the degree of recovery observed in the $O^{0.3}$ plants from D^0 to D^1 was higher than in the O^0 and $O^{0.1}$ plants, q_N was increased by a higher O_3 concentration ($O^{0.3}$). Additionally, in the O^0 plants, F_v/F_m did not change from D^{10} onward (Figs. 2–3A, B and 4A). Consequently, it appeared that q_I disappeared in the O^0 plants during a 10 min period of dark adaptation. Thus, because F_v/F_m decreased in the O^0 plants prior to D^5, it is difficult to distinguish the effects of O_3 and other factors (e.g., light) prior to this time point. As F_v/F_m recovered in the $O^{0.1}$ plants (increased) from D^{10} to D^{20}, the xanthophyll cycle-dependent quenching (fast relaxation phase) of q_I would also have been increased during that time. Consequently, if the dark adaptation period is greater than 20 min, the effects of O_3 on the fast relaxation phase of q_I will disappear. However, when only the effects on D1 protein inactivation are to be evaluated, leaves must be maintained for more than 20 min in the

dark. In the $O^{0.3}$ plants, F_v/F_m decreased as the dark adaptation period progressed from D^5. Therefore, if the dark adaptation period is too long, F_v/F_m might differ from the value obtained immediately after O_3 exposure.

The results of this experiment imply that the optimum dark adaptation period for evaluating F_v/F_m of PSII in O_3-exposed rice leaves is 10 min because the effect of O_3 is maximal at this time, and the effects of other factors on F_v/F_m disappear.

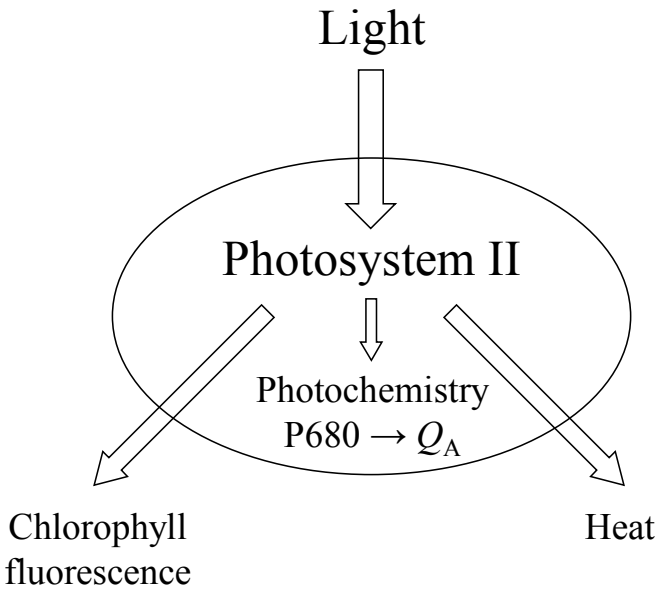


Fig. 2–1. A model of possible fate of light energy that is absorbed by photosystem II in green plants (redrawn from Baker, 2008). Light energy absorbed by chlorophylls associated with PSII can be used to photochemistry, heat loss, or chlorophyll fluorescence.

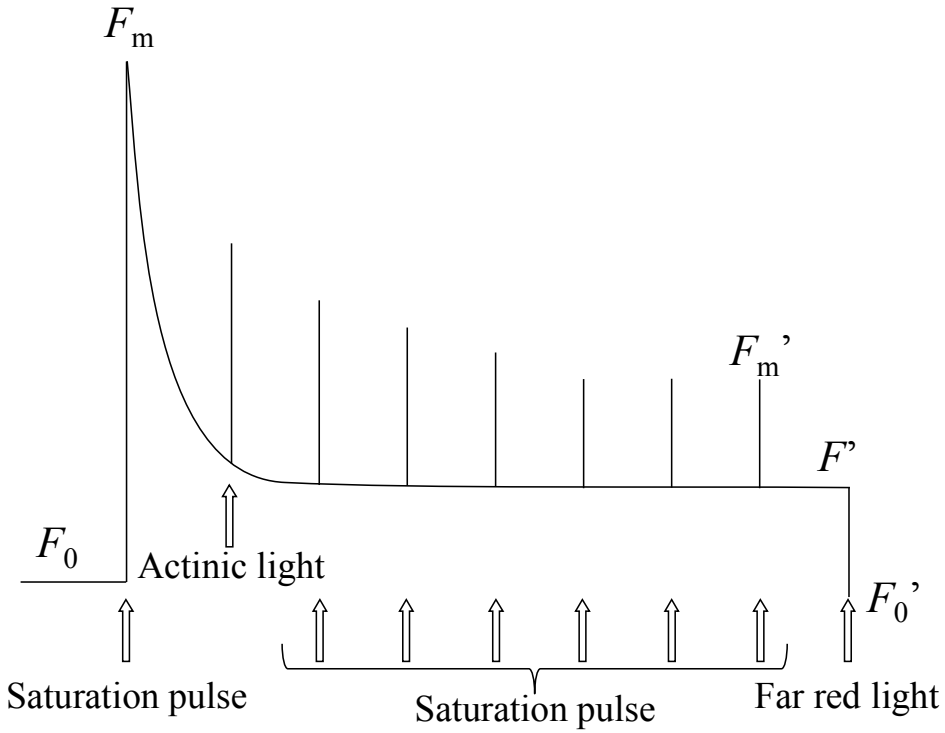


Fig. 2–2. Change of fluorescence intensity during chlorophyll fluorescence measurement (redrawn from Baker, 2008). F_0, minimum fluorescence in the dark-adapted state; F_m, maximum fluorescence in the dark-adapted state; F', steady fluorescence; F_0', minimum fluorescence at the given PPFD; F_m', maximum fluorescence at the given PPFD.

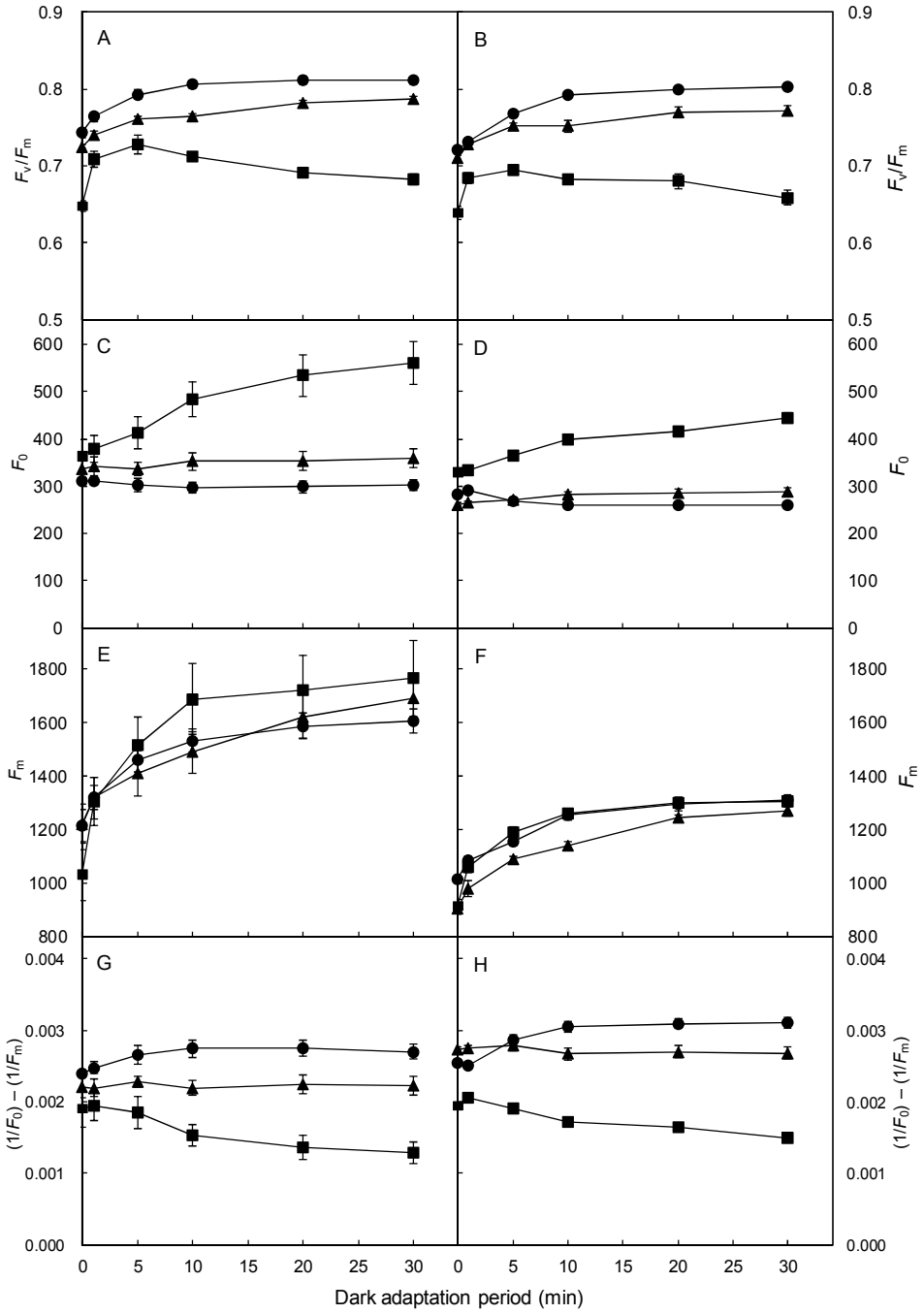


Fig. 2–3. Effects of O_3 on the maximum quantum efficiency of PSII (F_v/F_m), minimum fluorescence (F_0), maximum fluorescence (F_m), and $(1/F_0) - (1/F_m)$ under different dark adaptation periods in rice leaves (Exp. 1). The fluorescence parameters for A, C, E, and G were obtained using LI-6400-40 fluorometer, whereas those for B, D, F, and H were obtained using MINI-PAM fluorometer. Vertical bars represent standard errors of the mean (n = 5). ●, ▲, ■: 0, 0.1, and 0.3 $cm^3\ m^{-3}$ O_3.

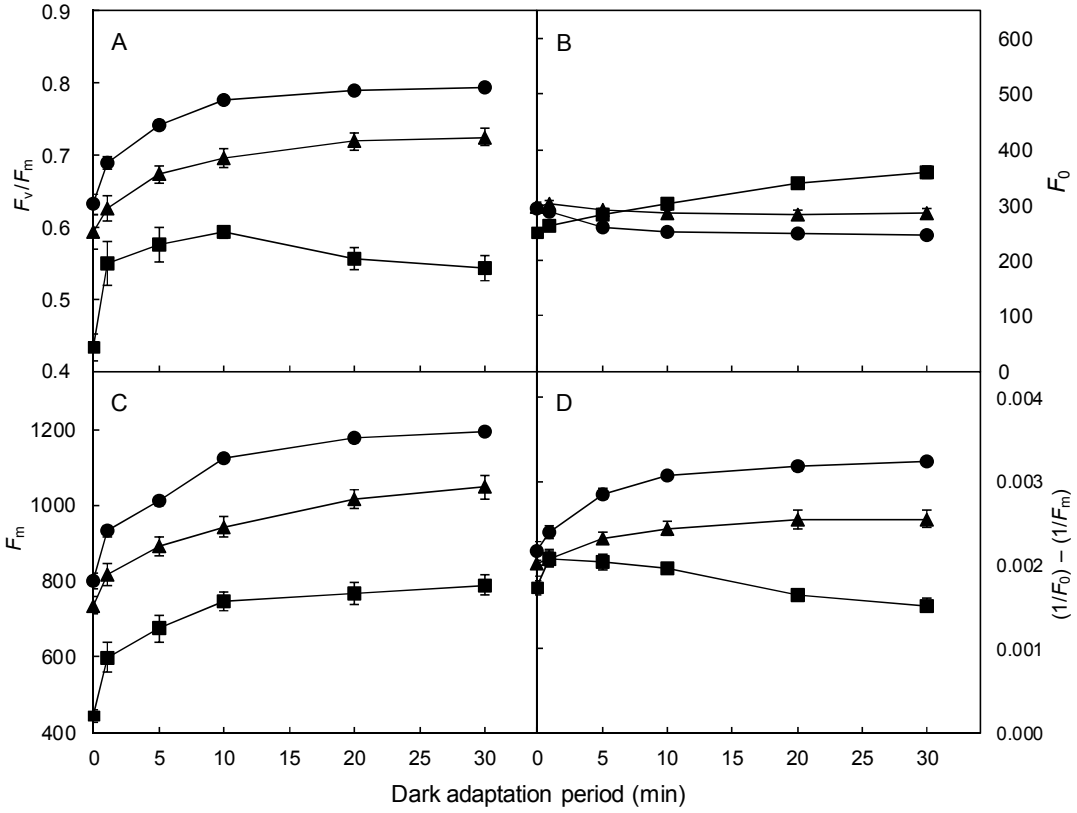


Fig. 2–4. Effects of O_3 on the maximum quantum efficiency of PSII (F_v/F_m), minimum fluorescence (F_0), maximum fluorescence (F_m), and $(1/F_0) - (1/F_m)$ under different dark adaptation periods in rice leaves (Exp. 2). The fluorescence parameters were obtained using MINI-PAM fluorometer. Vertical bars represent standard errors of the mean ($n = 5$). ●, ▲, ■: 0, 0.1, and 0.3 $cm^3\ m^{-3}$ O_3.

Table 2-1. The definition and physiological relevance of chlorophyll fluorescence parameters used in this study (Baker, 2008).

Parameter	Definition	Physiological relevance
F_v/F_m	Maximum quantum efficicency of PSII	Maximum efficiency at which light absorbed by PSII is used for reduction of Q_A.
F_q'/F_m'	Operating quantum efficiency of PSII	At a given PPFD, this parameter provides an estimate of the quantum yield of linear electron flux through PSII.
F_v'/F_m'	Maximum quantum efficicency of PSII at a given PPFD	Operating quantum efficiency of PSII if all the PSII centers were 'open' (Q_A oxidized).
q_p	Photochemical quenching coefficient of PSII	Proportion of PSII centers that are 'open' (Q_A oxidized).
q_N	Nonphotochemical quenching coefficient of PSII	Apparent rate constant for heat loss from PSII.

Table 2–2. Statistical analyses of the effects of O_3 and dark adaptation period on chlorophyll fluoresence parameters in rice leaves.*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. n.s., not significant by two-way ANOVA. F_0, minimum fluorescence in the dark-adapted state; F_m, maximal fluorescence in the dark-adapted state; F_v/F_m, maximal quantum efficiency of PSII.

Experiment	Factor	F_v/F_m	F_0	F_m	$(1/F_0) - (1/F_m)$
Exp. 1 (LI-6400XT)	O_3	***	***	n.s.	***
	dark adaptation period	***	*	***	n.s.
	O_3×dark adaptation period	***	**	n.s.	n.s.
Exp. 1 (MINI-PAM)	O_3	***	***	***	***
	dark adaptation period	***	***	***	n.s.
	O_3×dark adaptation period	***	***	n.s.	***
Exp. 2 (MINI-PAM)	O_3	***	***	***	***
	dark adaptation period	***	**	***	***
	O_3×dark adaptation period	*	***	n.s.	***

Table 2–3. Statistical analyses of the chlorophyll fluoresence parameters in rice leaves between control plants (O^0) and O_3-treated plants ($O^{0.1}$ or $O^{0.3}$) under different dark adaptation periods .*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. n.s., not significant by Turkey's test. F_0, minimum fluorescence in the dark-adapted state; F_m, maximal fluorescence in the dark-adapted state; F_v/F_m, maximal quantum efficiency of PSII.

Experiment	Parameter	O_3 (cm^3 m^{-3})	Dark adaptation period (min)					
			0	1	5	10	20	30
Exp. 1 (LI-6400XT)	F_v/F_m	$O^{0.1}$	n.s.	n.s.	n.s.	***	**	*
		$O^{0.3}$	***	**	***	***	***	***
	F_0	$O^{0.1}$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O^{0.3}$	n.s.	n.s.	*	**	***	***
	F_m	$O^{0.1}$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O^{0.3}$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	$(1/F_0) - (1/F_m)$	$O^{0.1}$	n.s.	n.s.	n.s.	*	n.s.	n.s.
		$O^{0.3}$	n.s.	n.s.	*	***	***	***
Exp. 1 (MINI-PAM)	F_v/F_m	$O^{0.1}$	n.s.	n.s.	n.s.	**	*	*
		$O^{0.3}$	***	***	***	***	***	***
	F_0	$O^{0.1}$	**	**	n.s.	n.s.	n.s.	n.s.
		$O^{0.3}$	***	***	***	***	***	***
	F_m	$O^{0.1}$	*	*	*	**	n.s.	n.s.
		$O^{0.3}$	*	n.s.	n.s.	n.s.	n.s.	n.s.
	$(1/F_0) - (1/F_m)$	$O^{0.1}$	**	**	n.s.	**	*	**
		$O^{0.3}$	***	***	***	***	***	***
Exp. 2 (MINI-PAM)	F_v/F_m	$O^{0.1}$	n.s.	n.s.	*	***	**	**
		$O^{0.3}$	***	**	***	***	***	***
	F_0	$O^{0.1}$	n.s.	n.s.	**	**	**	*
		$O^{0.3}$	**	*	*	***	***	***
	F_m	$O^{0.1}$	n.s.	n.s.	*	***	**	**
		$O^{0.3}$	***	***	***	***	***	***
	$(1/F_0) - (1/F_m)$	$O^{0.1}$	n.s.	n.s.	**	***	***	***
		$O^{0.3}$	n.s.	n.s.	***	***	***	***

Chapter 3

Effects of O_3 and CO_2 on Photosynthesis in Leaves of Paddy Rice

Section 1

Effects of the Interaction between O_3 and CO_2 on Gas Exchange, Photosystem II and Antioxidants in Leaves of Paddy Rice

3 – 1 – 1. Introduction

To maintain rice yield, it is necessary to understand the role of O_3 and CO_2 in both carbon fixation and photochemical reactions as shown in Chapter 1. Therefore, the author examined the effects of O_3 and CO_2 on net photosynthetic rate (P_N), stomatal conductance (g_s) and photosystem II (PSII) at the vegetative and reproductive stages of rice development to determine their interactive effects. Furthermore, the author measured the contents of ascorbic acid and glutathione as major antioxidants in rice.

3 – 1 – 2. Materials and methods

Plant materials and gas exposure

On May, Japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown as shown in Chapter 2. Plants were cultivated in a natural-light glasshouse with ventilation from early May to late August when the gas exposures began. Just after the full expansion of the 7th leaves (Haun index = 7.0, Haun, 1973) or the 16th leaves (flag leaf, Haun index = 16.0), the plants were transferred to a natural-light gas-exposure chamber (S-2003A; Koito Industries, Yokohama, Japan) and kept at 28/23°C (12-h day/12-h night), 60% RH and 400 cm^3 m^{-3} CO_2. Just after the full expansion of the 8th leaves or at heading, 5 h gas exposure treatments (08:00–13:00; local time) were applied under several combinations of O_3 and CO_2: $O^0 + C^{400}$ (control = clean air), $O^0 + C^{800}$, $O^{0.1}$ or $O^{0.3} + C^{400}$,

and $O^{0.1}$ or $O^{0.3}$ + C^{800}. O_3 was supplied using a high voltage ozone generator with dry air (MO-5A; Ozone System, Tokyo, Japan), and CO_2 was supplied from cylinders containing liquid CO_2. These gases were injected and measured as shown in Chapter 2. After the 5 h gas exposure, all the plants were kept in the same chamber for 3 d under 28/23°C 60% RH and 400 cm^3 m^{-3} CO_2 (O^0 + C^{400}).

Gas-exchange measurements

To compare the responses of leaves at different growth stages, *in situ* gas-exchange measurements were made on the 8th leaves (vegetative state of plant development) or the 16th leaves (heading) on the main stem. Measurements were made just before gas exposure (BE: 1–0 h before), during gas exposure (DE: 4–5 h from the start of gas exposure), just after gas exposure (AE-0: 0.1–1.1 h after) and 1 and 3 d after gas exposures (AE-1, AE-3). Two portable photosynthesis systems (LI-6400XT; LI-COR, Lincoln, NE, USA) were used, with 5 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 μmol m^{-2} s^{-1} PPFD (mixed light from red and blue LEDs). Gas-exchange measurements of the 8th leaves also were conducted at 23°C leaf temperature and 1.5 kPa VPD in darkness (1–2 h after the beginning of dark period) to obtain respiration rate (R_D).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were conducted simultaneous with the gas-exchange measurements using a fluorometer (LI-6400-40; LI-COR, Lincoln, NE, USA) attached to LI-6400 system, with the same replicate plants that were used for photosynthesis. Chlorophyll fluorescence measurements were made by applying 0.1, 7,000 and 1,500 μmol m^{-2} s^{-1} of measuring light, saturating pulse (flash) and actinic light, respectively. Before the fluorescence measurements, the leaves were kept in the dark for 10 min and then the minimum (F_0) and maximum (F_m) fluorescence were determined by irradiating the measuring light and saturating pulses. Thereafter,

the steady fluorescence (F'), and maximum fluorescence (F_m') in the steady state were determined under actinic light irradiation. The minimum fluorescence in the steady state (F_0') was determined during a brief interruption of actinic light irradiation in the presence of far-red light. The maximum quantum efficiency (F_v/F_m), operating efficiency (F_q'/F_m'), maximum efficiency at the given PPFD (F_v'/F_m') and the photochemical (q_p) and non-photochemical (q_N) quenching coefficients of PSII were obtained using the equations as shown in Chapter 1 (Baker, 2008, Sonoike, 2009):

Antioxidant measurements

Separate sets of plants were exposed to combined O_3 and CO_2 and used for measurement of antioxidant concentrations. The 7th and 8th leaves at vegetative stage or the 15th and 16th (flag) leaves at heading were sampled at BE, AE-0, AE-1 and AE-3 with 4 replicate plants (n = 4) in each chamber. Immediately after the measurements of fresh weight (FW), leaves were frozen in liquid N_2 and ground with a pestle and mortar by adding metaphosphoric acid to obtain leaf extracts. Because of the limited numbers of replicate plants, the amount of ascorbic acid (reduced form: AA; oxidized form: DHA) for the 7th- or 15th-leaf extract was determined by the hydrazine method (Fujita and Yamada, 2006) on a spectrophotometer (Ubest-30; JASCO Co., Tokyo, Japan), and the amount of glutathione (reduced form: GSH; oxidized form: GSSG) for the 8th- or 16th-leaf extract by the enzymatic recycling method (Mano et al., 2009) on a microplate reader (MTP-450 (Lab); Corona Electric Co., Ibaraki, Japan). The hydrazine 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 standard reagents used were L(+)-ascorbic acid (Kanto Chemical Co., Inc., Japan), glutathione (oxidized form, Wako Pure Chemical Ind., Ltd., Japan), and an enzyme kit consists of glutathione reductase, 5-5'-dithiobis(2-nitrobenzoic acid) and NADPH

(NWLSSTM Glutathione Assay, Northwest Life Science Specialities, LLC, USA). The redox states (RDS) of ascorbic acid and glutathione were calculated as follows: AA/(AA + DHA) and GSH/(GSH + GSSG), respectively.

Statistical analysis

All data were subjected to a two-way analysis of variance (ANOVA) with Excel Statistics 2010 for Windows software package (Social Survey Research Information Co., Tokyo, Japan). The significance among treatments in each time interval was determined by Tukey's test. Appropriate standard errors of the means (SE) were calculated, and the results are presented as line graphs.

3 – 1 – 3. Results

Effects of O_3 and CO_2 on photosynthesis in the 8th leaves

The results for gas exchange and PSII are shown in Fig. 3–1–1. At DE, the P_N of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants decreased to 72, 45, and 91%, respectively, of those at BE. At AE-0, these further decreased to 49, 38, and 46%, respectively, of those at BE. However, in clean air ($O^0 + C^{400}$), the plants began to recover from O_3-induced decline, and at AE-1, the P_N values were 78, 60, and 83%, respectively, of those at BE. The P_N recovered further by AE-3, when there was no significant difference between the $O^{0.1} + C^{400}$ and $O^0 + C^{400}$ plants. However, the inhibition of the P_N in the $O^{0.3} + C^{400}$ plants remained low from AE-1 to AE-3, and the P_N of the $O^{0.3} + C^{400}$ at AE-3 plants was 60% of that at BE (Fig. 3–1–1A). In the $O^0 + C^{800}$, $O^{0.1} + C^{400}$, $O^{0.1} + C^{800}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants, the g_s substantially decreased compared to that of the $O^0 + C^{400}$ plants. With the same O_3 concentration, the g_s of the C^{400} and C^{800} plants were similar at DE. At AE-0, the g_s stayed at the same level as those at DE, except that of the $O^{0.1} + C^{400}$ plants, which decreased further. The g_s recovered almost completely between AE-1 and AE-3, when there was no significant difference between any of the treatments, except the $O^{0.3} + C^{400}$ plants (Fig. 3–1–1B). As shown in Table 3–1–1, there were significant, direct effects of O_3 and CO_2 and also,

interaction between these two factors on the P_N and g_s of the 8th leaves at DE. At AE-0, the effect of CO_2 was diminished because plants were kept in an air of C^{400}. However, a significant effect remained for CO_2 on the P_N and g_s remained at AE-1 and AE-3 because the leaves received $O^{0.1}$ treatment recovered well while those received $O^{0.3}$ treatment were severely damaged.

The chlorophyll fluorescence measurements revealed responses similar to the P_N responses (Fig. 3–1–1C, D, E, F, G). The F_v/F_m of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased to 92, 86, and 95%, respectively, of that at BE. At AE-0, they decreased further to 91, 81, and 89%, respectively, of those at BE. In the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants, the F_v/F_m recovered at AE-1, but that of the $O^{0.3} + C^{400}$ plants was 92 and 93% of the initial values on AE-1 and AE-3, respectively (Fig. 3–1–1C). The F_q'/F_m' decreased at DE and AE-0, as did F_v/F_m. This damage was slightly reversed on AE-1, but the values for the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants were still decreased to 70, 62, and 81%, of that at BE. At AE-3, the inhibition disappeared completely in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants, but the activity of the $O^{0.3} + C^{400}$ plants was still 28% lower than that of the control plants ($O^0 + C^{400}$) (Fig. 3–1–1D). The F_v'/F_m' of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants at DE decreased to 81, 65, and 80%, respectively, of those at BE, but there was no significant difference between any of the treatments, except for the $O^{0.3} + C^{400}$ plants, from AE-0 to AE-3. On the other hand, the F_v'/F_m' of the $O^{0.3} + C^{400}$ plants was 73, 80 and 80% of the initial values at AE-0, AE-1 and AE-3, respectively (Fig. 3–1–1E). The q_p decreased in the $O^{0.3} + C^{400}$ plants at DE and in the $O^{0.3} + C^{400}$ and $O^{0.1} + C^{400}$ plants at AE-0. The q_p of the $O^{0.1} + C^{400}$, $O^{0.1} + C^{800}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants decreased to 77, 88, 77, and 83%, respectively, of the respective initial values at AE-1, but there was no significant difference between any of the treatments on AE-3 (Fig. 3–1–1F). The q_N of the $O^{0.3} + C^{400}$ plants was slightly high compared to that of the control at AE-3 (Fig. 3–1–1G). As a whole, the effects of O_3 and CO_2 on parameters of PSII were similar to the trends observed for P_N and g_s, but O_3 and CO_2 had less effect on q_p and q_N (Table 3–1–1).

The R_D of the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants at AE-0 increased to 147 and 234%,

respectively, of the respective initial values (Fig. 3–1–2A). The g_s in darkness of the $O^{0.3} + C^{400}$ plants at AE-0 increased tenfold compared to that of the control ($O^0 + C^{400}$), and this tendency was maintained until AE-3 (Fig. 3–1–2B). Statistical analyses indicated that the effects of O_3 and CO_2 on R_D and g_s in darkness at AE-0 and AE-1 were similar to those of P_N and g_s in light but the R_D recovered almost completely at AE-3 (Table 3–1–1).

Effects of O_3 and CO_2 on photosynthesis in the 16th (flag) leaves

As shown in Fig. 3–1–3, the P_N of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased at DE and AE-0, and recovered at AE-1 and AE-3. However, the P_N of the $O^{0.3} + C^{400}$ plants at AE-3 decreased to only 91% of that at BE (Fig. 3–1–3A). The g_s of all treatments decreased at DE, but this decrease was completely reversed at AE-1 (Fig. 3–1–3B). The F_v/F_m did not differ significantly among treatments at any measurement time (Fig. 3–1–3C). The F_q'/F_m' of the $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased at AE-0, but there was no significant difference between the other treatments (Fig. 3–1–3D). The F_v'/F_m' of the $O^{0.3} + C^{400}$ plants decreased to 86% of that at BE, and this occurred in the $O^{0.3} + C^{800}$ plants at AE-0 as well. However, there was no significant difference between any of the plants from AE-1 to AE-3 (Fig. 3–1–3E). The q_p in the $O^{0.3} + C^{400}$ plants decreased to 84 and 88% of the initial values at DE and AE-0, respectively. However, no significant difference was observed between the control and the $O^{0.3} + C^{400}$ plants at AE-1 (Fig. 3–1–3F). Table 3–1–1 showed that the 16th leaves had similar trend with the 8th leaves in the P_N but the g_s was less affected and the PSII was almost unaffected by O_3 and CO_2 as seen in Fig. 3–1–3.

Effects of O_3 and CO_2 on the antioxidants

At BE, the total ascorbic acid (Total), reduced ascorbic acid (AA) and dehydroascorbic acid (DHA) contents [mmol kg^{-1}(FW)] and the redox state (RDS) of ascorbic acid (RDS = AA/Total) in the 7th leaves were 5.73, 2.90, 2.83 and 0.50, respectively, and those in the 15th leaves were 8.46, 0.78, 7.68, and 0.10, respectively. Table 3–1–2 shows that the total ascorbic acid content in the 7th

leaves was not affected at AE-0, but the contents of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants decreased significantly at AE-1 compared to those at BE. The RDS of ascorbic acid in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants at AE-1 decreased to 55, 51, and 55%, respectively, of those at BE. The $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants recovered from this decrease by AE-3, but the $O^{0.3} + C^{400}$ plants did not. The total ascorbic acid content in the 15th leaves was not affected by gas exposures. The RDS of ascorbic acid in the $O^{0.3} + C^{400}$ plants decreased at AE-1, but recovered at AE-3. There was no interactive effect between O_3 and CO_2 in both 7th and 15th leaves for any parameter, except RDS of the 7th leaves at AE-3.

The total glutathione (Total), reduced glutathione (GSH) and oxidized glutathione (GSSG) contents [μmol kg^{-1}(FW)] and the RDS of glutathione (RDS = GSH/ Total) in the 8th leaves at BE were 66.0, 48.5, 17.5, and 0.72, respectively, and those in the 16th (flag) leaves were 139.1, 117.3, 21.8, and 0.84, respectively. Table 3–1–3 shows that the total glutathione content in the 8th leaves in the $O^{0.3} + C^{400}$ plants at AE-3 increased to 165% of BE. The decrease in RDS and concomitant increase in GSSG occurred with O_3 exposures in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants at AE-0 and AE-1. However, these were recovered by AE-3. The total glutathione content in the 16th leaves of the $O^{0.3} + C^{400}$ plants at AE-1 increased to 144% of the BE, and that of the $O^{0.3} + C^{800}$ plants at AE-3 was also higher than the BE. As seen in ascorbic acid, there was no interactive effect between O_3 and CO_2 in both 8th and 16th leaves, except total glutathione and GSH of the 16th leaves at AE-1 (Table 3–1–3).

3 – 1 – 4. Discussion

Consistent with previous observation in rice leaves (Imai and Kobori, 2008), P_N was inhibited by $O^{0.1}$ and $O^{0.3}$ but ameliorated by C^{800} in the 8th leaves (Fig. 3–1–1A), and one of the causal factors was stomatal closure due to elevated CO_2, which limited the O_3 intake (McKee et al., 1997, Mulholland et al., 1997, Booker and Fiscus, 2005). At the same O_3 concentration, g_s was similar irrespective of CO_2 concentration. However, at the same CO_2 concentration, g_s was lower at

higher O_3 concentration (Fig. 3–1–1B). Therefore, the limitation of O_3 uptake through the stomata cannot fully explain the O_3-induced decrease in P_N and its amelioration by elevated CO_2, as shown by the larger decline of P_N under $O^{0.3}$ than under $O^{0.1}$ at the same C_i (Imai and Kobori, 2008). Mesophyll dysfunction, including the inactivation of Rubisco (Ishioh and Imai, 2005) and/or the photosystem (Rai and Agrawal, 2008), could be other reasons. The O_3-induced decrease in P_N started to attenuate at AE-1, and almost disappeared at AE-3, except in the $O^{0.3} + C^{400}$ plants (Fig. 3–1–1A). A similar situation was found in the case of g_s, and, therefore, this section assumed that one reason why P_N did not recover for a long time after gas exposure, as in the $O^{0.3} + C^{400}$ plants, was stomatal dysfunction (Fig. 3–1–1B), in which O_3 adversely affects the osmotic adjustment of the guard cells toward the inhibition of the K^+ channel (Torsethaugen et al., 1999). In accordance with the observation by Imai and Kobori (2008), the abnormally high g_s in the $O^{0.3} + C^{400}$ plants in darkness (Fig. 3–1–2B), which was equivalent to that under illumination (Fig. 3–1–1B) supports the above-mentioned assumption.

Though the effects of O_3 on parameters of PSII were not as large compared to those of gas exchange, the F_q'/F_m' in the 8th leaves of the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased from DE to AE-1, but that of the $O^{0.3} + C^{400}$ plants decreased until AE-3 (Fig. 3–1–1D). Similarly, the F_v'/F_m' in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased from DE to AE-0, but that of the $O^{0.3} + C^{400}$ plants decreased until AE-3 (Fig. 3–1–1E). On the other hand, the q_p of the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants decreased compared to that of the control ($O^0 + C^{400}$) at AE-0, and the q_p of the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants decreased at AE-1 (Fig. 3–1–1F). Therefore, the initial factor of the decrease in F_q'/F_m' in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{800}$ plants at DE and AE-0 was ascribed mainly to the F_v'/F_m', and the second factor continued to AE-1 was the q_p: O_3 first disrupted the PSII, and then adversely affected the downstream of plastoquinone A (Q_A) in photosynthetic electron transport. The decrease in F_v'/F_m' from DE to AE-3 by the $O^{0.3} + C^{400}$ plants indicated severe disruption of PSII by O_3. The P_N of the $O^{0.3} + C^{400}$ plants did not recover for a long time due to the damage sustained by PSII. Consequently, as the PSII is one of the most

vulnerable parts to O_3 exposure, the recovery of PSII from the O_3-induced damage also indicates the recovery in other parameters of photosynthesis.

In the 16th leaves, P_N decreased as a result of O_3 exposure, and the reduction due to O_3 was ameliorated by C^{800} (Fig. 3–1–3A), as also observed in the 8th leaves (Fig. 3–1–1A). However, the damage sustained by the 16th leaves was less, and they recovered faster than the 8th leaves: the decreased P_N in the $O^{0.3} + C^{400}$ plants recovered almost completely at AE-3. Also, the disruption of the PSII in the $O^{0.3} + C^{400}$ plants was less and the 16th leaves (Fig. 3–1–3C, D, E, F) recovered faster than in the 8th leaves (Fig. 3–1–1C, D, E, F). Since the F_q'/F_m' in the $O^{0.3} + C^{800}$ plants decreased at AE-0 and that of the $O^{0.3} + C^{400}$ plants decreased at DE, the decrease in the former treatment was ascribed to the decrease in F_v'/F_m', and the decrease in the latter treatment to decreases in both the F_v'/F_m' and the q_p. Only the PSII was suppressed in the $O^{0.3} + C^{800}$ plants, whereas both the PSII and the downstream reactions of Q_A were suppressed in the $O^{0.3} + C^{400}$ plants. However, as the F_v/F_m values were not significantly different between the control and $O^{0.3}$ plants, the damage of the PSII in the 16th leaves was recovered faster than that in the 8th leaves. Also, the q_N of the $O^{0.3}$ plants increased more than in the control at AE-3 both in the 8th and 16th leaves, indicating the treatments with more damage and late recovery consumed an excessive energy. This manifested as heat dissipation derived from the decreased energy consumption for the carbon fixation reaction.

Rao et al. (1995) reported in wheat that the activation of the ascorbate-glutathione cycle is induced by O_3 exposure, based on increases in DHA and GSSG. However, McKee et al. (1997) found no increase in ascorbic acid or glutathione content caused by elevated CO_2 during fumigation of wheat with air containing moderately elevated O_3. In this section, the RDS of ascorbic acid and glutathione decreased as a result of O_3 exposure where photosynthesis was severely impeded, but these changes occurred at different times, because the recovery of AA was slower than that of GSH (Tables 3–1–2 and 3). This section considered that, (1) ascorbic acid and glutathione might detoxify the ROS in different way(s) besides the ascorbate-glutathione cycle,

and/or (2) changes in RDS did not seem to harmonize irrespective of active ascorbate-glutathione cycle operation, since each one molecule of DHA and GSH react but their amounts are very different in the rice leaves. Indeed, the amount of ascorbic acid in rice leaves was far greater than that of glutathione (Inada et al., 2008). Furthermore, the elevated CO_2 did not compensate for the decline of these antioxidant levels (Tables 3–1–2 and 3). Therefore, changes in antioxidant levels in our experiment did not explain the amelioration of photosynthesis by elevated CO_2 to O_3 exposure.

In the rice plant, Ishioh et al. (2005) observed that at the reproductive stage, the detrimental effects of O_3-fumigation on the P_N and dry weight were smaller than vegetative stage. In this section, the responses of the P_N in the 8th and the 16th (flag) leaves to O_3 and CO_2 were substantially different (Fig. 3–1–1, Table 3–1–1), probably due to differences in leaf thickness and inclination angle. The leaves become thicker with increasing height of the leaf position (Hoshikawa, 1989), i.e. the 16th leaves are thicker than the 8th leaves. Because the concentration of antioxidants per FW is higher in the 16th leaves (Tables 3–1–2 and 3), it is easy to anticipate that the inhibition of the P_N and related processes in those leaves is less than in the 8th leaves due to higher contents of antioxidants per unit leaf area in the 16th leaves. On the other hand, the inclination angle of rice leaves becomes steeper at later growth stages (Ito et al., 1973). Because the inclination angle of the 16th leaves is steeper than that of the 8th leaves, the amount of incident light on the 16th leaves is smaller than on the 8th leaves. Consequently, the probability of photoinhibition in the older (16th) leaves would be smaller than in the younger (8th) leaves, as reported by Heath (1994) in *Phaseolus vulgaris*. In wheat, Mulholland et al. (1997) observed that the O_3-induced inhibition of the P_N was less pronounced in the 8th (flag) leaves than in the 5th to 7th leaves, and they ascribed this to the greater content of active Rubisco and the absence of shade-acclimation of the flag leaves.

In summary, the photosynthesis mechanism and related processes in rice plants growing near urban areas occasionally sustain damage due to acute exposure to photochemical oxidants

(especially O_3), leading to a decrease in the potential yield. However, since O_3-induced injury is ameliorated by elevated CO_2, the injury may be reduced in the future because the background O_3 level does not rise significantly. Therefore, it is important to ascertain whether such responses actually occur during the lifecycle of the rice plants with elevated CO_2 (Olszyk and Wise, 1997), and to analyze the plant hormones which mediate oxidative signal transduction (Morita and Tanaka, 2002, Baier et al., 2005).

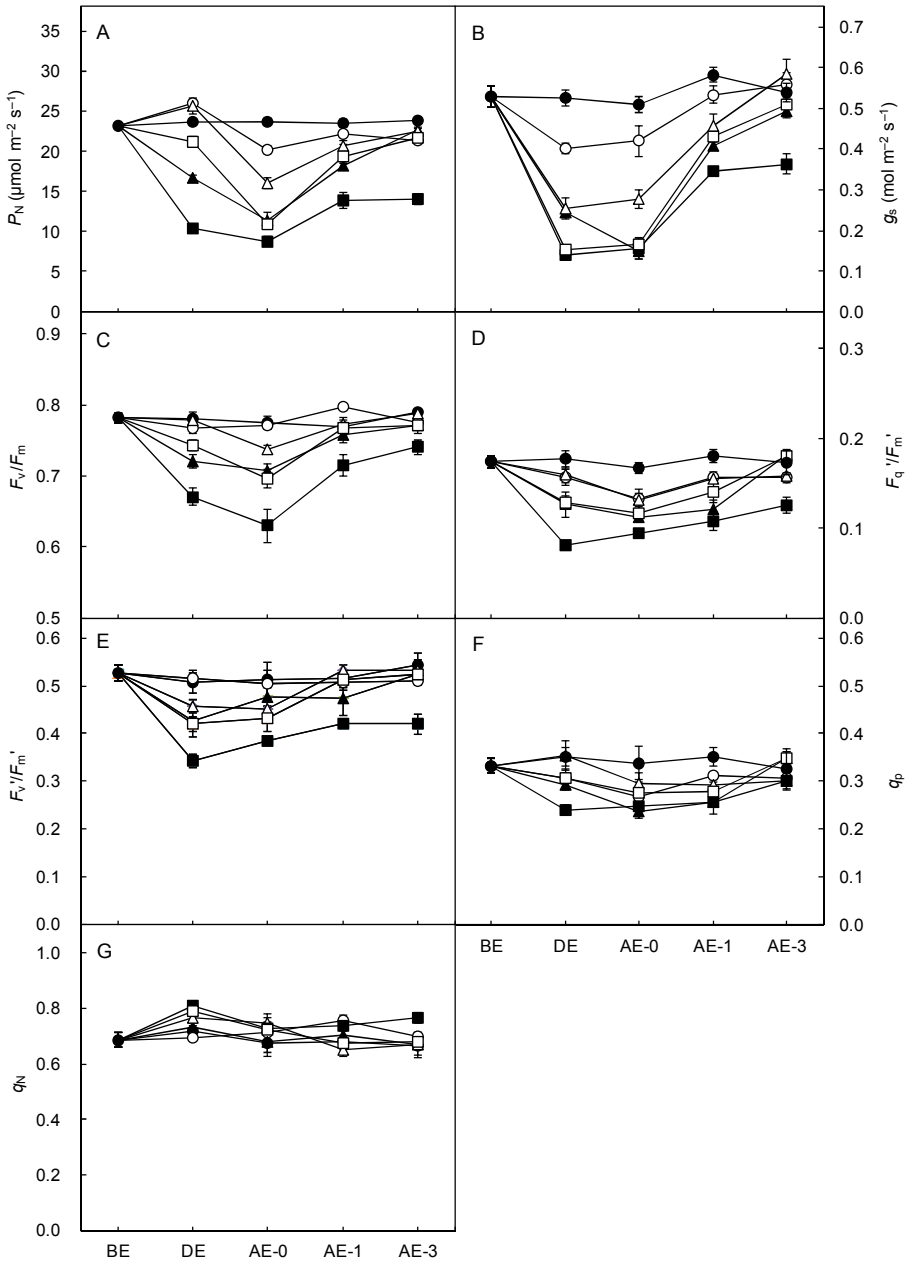


Fig. 3–1–1. Effects of O_3 and CO_2 on the net photosynthetic rate (P_N), stomatal conductance (g_s), and the maximum quantum efficiency (F_v/F_m), operating efficiency (F_q'/F_m'), maximum efficiency at 1,500 μmol m^{-2} s^{-1} PPFD (F_v'/F_m'), photochemical (q_p) and non-photochemical (q_N) quenching coefficients of PSII in the 8th leaves. Vertical bars represent standard errors of the means (n = 5). BE, DE, AE-0, AE-1 and AE-3 respectively denote before, during, immdeiately after, and 1 d and 3 d after gas exposure, respectively. ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

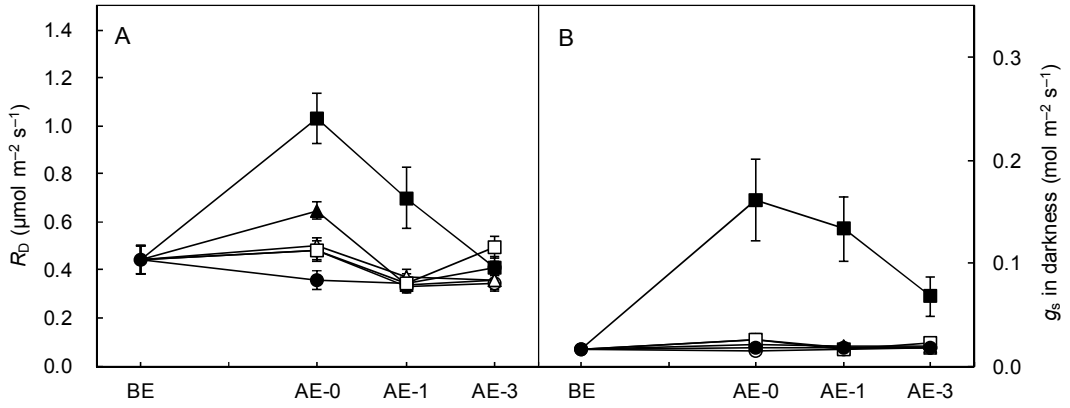


Fig. 3–1–2. Effects of O_3 and CO_2 on the respiration rate (R_D) and stomatal conductance (g_s) in darkness in the 8th leaves. Vertical bars represent standard errors of the means (n = 5). BE, AE-0, AE-1 and AE-3 respectively denote before, during, immediately after, and 1 d and 3 d after gas exposure, respectively. ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

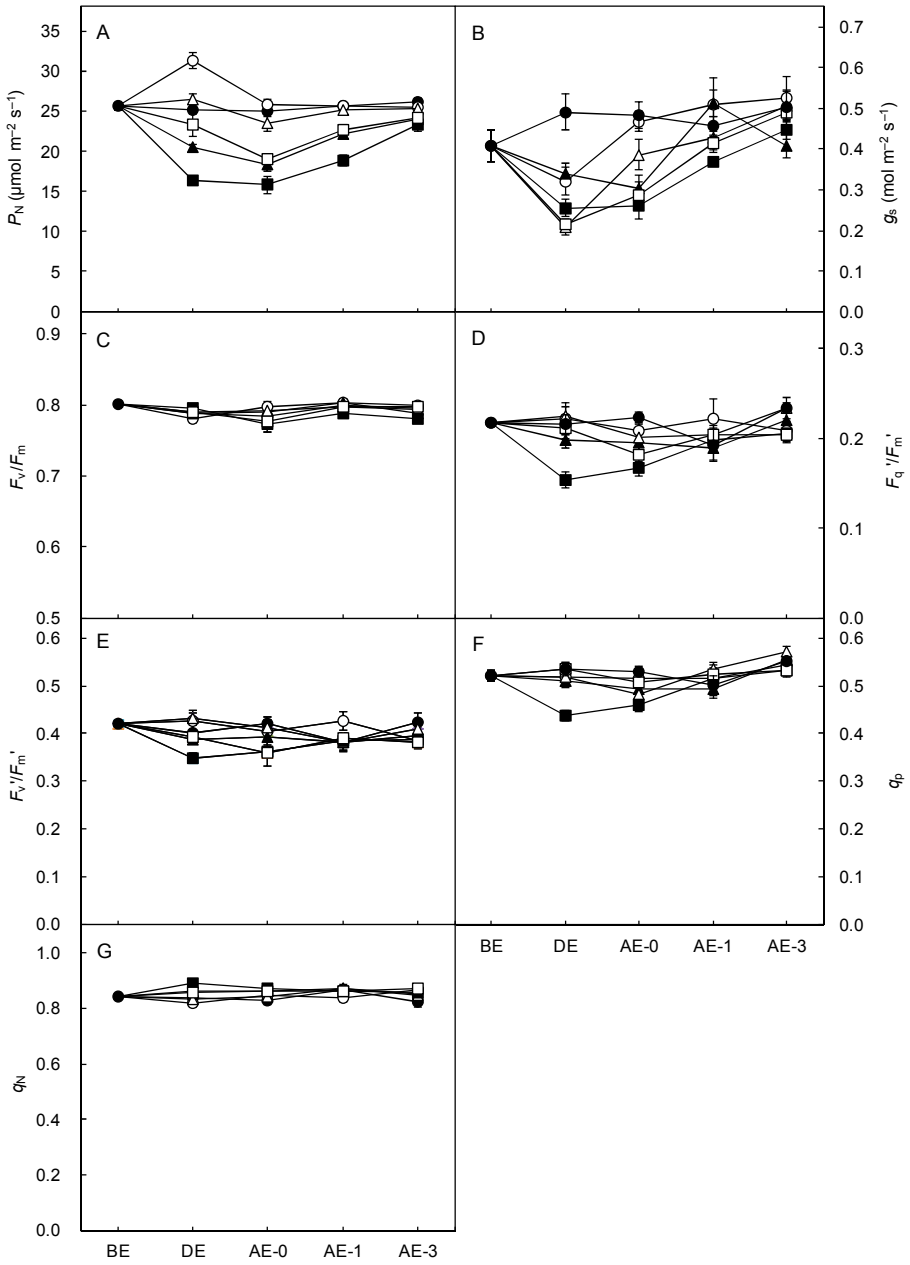


Fig. 3–1–3. Effects of O_3 and CO_2 on the net photosynthetic rate (P_N), stomatal conductance (g_s), and the maximum quantum efficiency (F_v/F_m), operating efficiency (F_q'/F_m'), maximum efficiency at 1,500 μmol m^{-2} s^{-1} PPFD (F_v'/F_m'), and photochemical (q_p) and non-photochemical (q_N) quenching coefficients of PSII in the flag leaves. Vertical bars represent standard errors of the means (n = 5). BE, DE, AE-0, AE-1 and AE-3 respectively denote before, during, immediately after, and 1 d and 3 d after gas exposure, respectively. ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

Table 3–1–1. Statistical analyses of the effects of O_3 and CO_2 on the gas exchange and chlorophyll fluorescence parameters of rice leaves. DE, AE-0, AE-1 and AE-3 respectively denote values obtained during, immediately after, 1 d, and 3 d after the gas exposure, respectively. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, n.s. - not significant, by two-way ANOVA. F_q'/F_m' - operating quantum efficiency of PSII; F_v/F_m - maximum quantum efficiency of PSII; F_v'/F_m' - maximum quantum efficiency at 1,500 μmol m^{-2} s^{-1} PPFD; g_s - stomatal conductance; P_N - net photosynthetic rate; q_N - non-photochemical quenching coefficient; q_P - phochemical quenching coefficient; R_D - respiration rate.

Leaf position	Time	Factor	P_N	g_s	F_v/F_m	F'_q/F'_m	F'_v/F'_m	q_P	q_N	R_D	g_s in darkness
8th	DE	O_3	***	***	***	***	***	*	*		
		CO_2	***	*	***	*	*	n.s.	n.s.		
		$O_3 \times CO_2$	***	**	***	*	n.s.	*	n.s.		
	AE-0	O_3	***	***	***	***	**	n.s.	n.s.	***	**
		CO_2	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	**	**
		$O_3 \times CO_2$	***	**	n.s.	**	n.s.	*	n.s.	***	**
	AE-1	O_3	***	***	**	***	n.s.	**	n.s.	*	**
		CO_2	**	n.s.	**	n.s.	*	n.s.	n.s.	n.s.	**
		$O_3 \times CO_2$	***	**	n.s.	**	n.s.	n.s.	*	*	**
	AE-3	O_3	***	**	**	n.s.	*	n.s.	n.s.	n.s.	*
		CO_2	***	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O_3 \times CO_2$	***	n.s.	*	***	**	n.s.	n.s.	n.s.	*
16th	DE	O_3	***	***	n.s.	*	*	n.s.	**		
		CO_2	***	***	n.s.	*	**	*	*		
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.		
	AE-0	O_3	***	***	n.s.	*	*	n.s.	n.s.		
		CO_2	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
	AE-1	O_3	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
		CO_2	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
		$O_3 \times CO_2$	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
	AE-3	O_3	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
		CO_2	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.		
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		

Table 3–1–2. Effects of O_3 and CO_2 on the ascorbic acid content [mmol kg^{-1}(FW)] and its redox state (RDS) in the 7^{th} and 15^{th} leaves. $n = 4$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, n.s. - not significant, by two-way ANOVA. AA - ascorbic acid; DHA - dehydroascorbic acid; Total = AA + DHA.

leaf position	O_3	CO_2	Just after gas exposure (AE-0)				1 d after gas exposure (AE-1)				3 d after gas exposure (AE-3)			
	[cm^3 m^{-3}]		Total	AA	DHA	RDS	Total	AA	DHA	RDS	Total	AA	DHA	RDS
7^{th}	O^0	C^{400}	5.20	2.86	2.34	0.57	6.69	3.36	3.33	0.50	5.58	3.13	2.44	0.56
		C^{800}	6.71	3.60	3.11	0.54	6.48	3.22	3.25	0.49	7.26	4.06	3.20	0.56
	$O^{0.1}$	C^{400}	5.28	2.96	2.32	0.60	4.08	1.17	2.91	0.28	2.60	1.45	1.14	0.56
		C^{800}	5.86	3.14	2.72	0.54	5.69	2.67	3.02	0.50	5.54	2.77	2.77	0.50
	$O^{0.3}$	C^{400}	4.25	2.26	1.98	0.56	2.98	0.75	2.09	0.26	2.81	0.73	2.08	0.25
		C^{800}	6.03	3.26	2.77	0.58	2.87	0.90	1.97	0.28	2.72	1.22	1.50	0.44
15^{th}	O^0	C^{400}	8.40	0.79	7.61	0.11	7.23	0.95	6.27	0.13	5.67	0.74	4.93	0.12
		C^{800}	7.21	0.80	6.41	0.11	6.64	0.90	5.74	0.13	5.80	0.50	5.30	0.11
	$O^{0.1}$	C^{400}	7.72	0.69	7.03	0.09	6.68	0.40	6.28	0.06	6.49	0.72	5.77	0.11
		C^{800}	7.19	0.86	7.04	0.11	6.50	0.52	5.98	0.10	6.47	0.81	5.66	0.13
	$O^{0.3}$	C^{400}	6.40	0.72	5.69	0.13	8.70	0.43	8.27	0.05	5.09	1.39	3.71	0.27
		C^{800}	6.86	0.68	6.17	0.10	5.96	0.57	5.40	0.09	6.30	0.58	5.72	0.11
ANOVA		Factor												
	7^{th}	O_3	n.s.	n.s.	n.s.	n.s.	***	***	*	*	**	***	n.s.	***
		CO_2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
	15^{th}	O_3	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		CO_2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3–1–3. Effects of O_3 and CO_2 on the glutathione content [mmol kg^{-1}(FW)] and its redox state (RDS) in the 8^{th} and flag (16^{th}) leaves. $n = 4$. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, n.s. - not significant, by two-way ANOVA. GSH - glutathione; GSSG - oxidized glutathione; Total = GSH + GSSG.

leaf position	O_3	CO_2	Just after gas exposure (AE-0)				1 d after gas exposure (AE-1)				3 d after gas exposure (AE-3)			
	[cm^3 m^{-3}]		Total	GSH	GSSG	RDS	Total	GSH	GSSG	RDS	Total	GSH	GSSG	RDS
8^{th}	O^0	C^{400}	69.4	51.7	17.7	0.75	65.6	49.8	15.7	0.71	58.7	39.7	19.0	0.62
		C^{800}	65.4	46.3	19.2	0.71	68.4	48.7	19.6	0.67	67.3	40.6	26.7	0.60
	$O^{0.1}$	C^{400}	68.2	29.1	39.1	0.39	62.9	28.1	34.8	0.40	66.4	40.9	25.5	0.56
		C^{800}	54.2	27.4	26.8	0.48	59.1	36.8	22.3	0.61	66.5	46.3	20.2	0.68
	$O^{0.3}$	C^{400}	71.6	30.8	40.8	0.40	57.6	24.6	33.0	0.42	109.1	78.2	31.0	0.73
		C^{800}	69.8	31.2	38.6	0.45	63.9	34.6	29.3	0.50	62.4	43.0	19.4	0.60
16^{th}	O^0	C^{400}	137.4	116.3	21.1	0.85	133.3	114.2	19.1	0.86	136.2	112.1	24.1	0.82
		C^{800}	135.2	115.2	20.0	0.85	134.0	113.5	20.5	0.84	134.3	113.6	20.7	0.84
	$O^{0.1}$	C^{400}	128.8	96.1	32.7	0.75	124.9	91.4	33.5	0.73	155.3	128.4	26.8	0.82
		C^{800}	132.6	115.8	16.8	0.87	147.2	124.4	22.8	0.83	139.0	113.4	25.5	0.82
	$O^{0.3}$	C^{400}	133.4	92.1	41.3	0.68	199.9	152.6	47.3	0.76	189.6	149.2	40.4	0.78
		C^{800}	138.7	100.2	38.4	0.71	140.9	93.3	47.7	0.66	175.8	131.0	44.8	0.75
ANOVA		Factor												
	8^{th}	O_3	n.s.	n.s.	**	**	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
		CO_2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	16^{th}	O_3	n.s.	n.s.	**	**	*	n.s.	**	*	***	*	**	n.s.
		CO_2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Section 2

Inclination Angle Affects O_3 Injury in the Flag Leaf of Paddy Rice

3 – 2 – 1. Introduction

The results of Chapter 3 Section 1 revealed that the damage of net photosynthetic rate (P_N), stomatal conductance (g_s) and photosystem II (PSII) by O_3 in the 8th (vegetative growth stage) and flag (16th, heading stage) leaves of rice differed substantially. In the 8th leaves, these parameters were inhibited severely by O_3 (0.1 and 0.3 cm^3 m^{-3}). Their inhibition at higher O_3 concentrations did not recover for 3 d after exposure to O_3, but the inhibition in the flag leaves was less severe and their recovery was faster. This difference is ascribable to their respective leaf thicknesses and leaf inclination angles. Regarding leaf thickness, these results have anticipated differences of O_3 inhibition because of the difference in antioxidant contents: the ascorbic acid and glutathione contents per unit of fresh weight (FW) in the 7th and 8th leaves (thinner than 15th and flag leaves) at the vegetative growth stage were lower than those in the 15th and flag leaves at the heading stage. However, the effects of leaf inclination angle on inhibition of photosynthesis-related processes by O_3 were not clarified. Therefore, the author examined the effect of the leaf inclination angle on the inhibition of P_N and PSII in the flag leaf by O_3 at the heading stage. This section described the comparison of inclination angle and relative light intensity on the adaxial surface, and the ascorbic acid contents of leaves at different positions on a stem to test the hypothesis that the difference in leaf inclination angle is related to the difference in injury by O_3.

3 – 2 – 2. Materials and methods

Plant materials and gas exposure

On April, japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown directly in Wagner pots (1/5000a), and plants were cultivated in a natural-light greenhouse as shown in Chapter 3 Section 1. Just after the full expansion of the flag (16th) leaves, the plants were transferred to a

natural-light gas-exposure chamber (S-2003A; Koito Industries, Ltd., Yokohama, Japan) and kept at 28/23°C (12-h day/12-h night), 60% RH and 400 cm^3 m^{-3} CO_2. At the heading stage, the middle portion of the flag leaf was arranged horizontally by attaching small, globular lead weights at the tip as shown in Fig. 3–2–1 (H plant). The relative light intensity at the middle portion of adaxial leaf surface relative to the light intensity at the top of main stem was 91.4% in H plants and 24.7% in the elect-leaf plants (control E plant). Both H and E plants were exposed to 0, 0.1, and 0.3 cm^3 m^{-3} O_3, expressed respectively as HO^0 and EO^0, $HO^{0.1}$ and $EO^{0.1}$, and $HO^{0.3}$ and $EO^{0.3}$ plants for 5 h in the day-time (08:00–13:00; local time) under isolated conditions with a minimum mutual shading. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 2. After the gas exposure treatment, all plants were kept in the same chamber for 3 d at 28/23°C, 60% RH, and 400 cm^3 m^{-3} CO_2 without O_3.

Gas-exchange measurements

Using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA), gas-exchange in the middle portion of flag leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment. Chlorophyll fluorescence measurements were made by applying 0.2, 7,000 and 1,400 μmol m^{-2} s^{-1} of measuring light, saturating pulse (flash) and actinic light, respectively. The measurement and calculation of chlorophyll fluorescence parameters were same as shown in Chapter 3 Section 1.

Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the flag leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 1.

Leaf inclination angle and relative light intensity measurements

The leaf inclination angle at the bottom and middle portion of leaf blade between the main stem (vertical axis) were measured using a protractor. The relative light intensity, which was the intensity relative to that of the top of main stem at the middle portion of leaf blade, was measured using a photon sensor attached to the fluorometer. Both measurements were conducted at the heading stage (for 16^{th} to 10^{th} leaves) each with five replicate plants.

Ascorbic acid measurements

At the heading time, each of four flag (16^{th}), 15^{th}, 14^{th}, and 13^{th} leaves were sampled and used for measurements of ascorbic acid (reduced form, AA; oxidized form, DHA). Immediately after measurements of the FW and leaf area, leaves were frozen in liquid N_2 and ground with a mortar and pestle by adding metaphosphoric acid to obtain leaf extracts. Then AA and DHA were determined by the hydrazine method (Fujita and Yamada, 2006) as shown in Chapter 3 Section 1. Using five separate set of plants, the leaf area was measured with an automatic area meter (LI-3100C, LI-COR Biosciences, Lincoln, NB, USA) and the leaf dry weight, after oven-drying at 80°C for 48 h.

Statistical analysis

Statistical analyses were conducted as shown in Chapter 3 Section 1.

3 – 2 – 3. Results

Effects of O_3 and leaf inclination angle on gas exchange parameters

Comparison of the HO^0 plants with the EO^0 plants in Fig. 3–2–2 revealed that the P_N and g_s were unaffected by attaching lead weights during the measurement periods. At AE-0, the P_N of the $EO^{0.1}$, $HO^{0.1}$, $EO^{0.3}$, and $HO^{0.3}$ plants decreased to 75, 71, 63, and 55%, respectively, of that at BE. Thereafter, the O_3-inhibition of P_N began to recover at AE-1: the $EO^{0.1}$, $HO^{0.1}$, $EO^{0.3}$, and $HO^{0.3}$

plants recovered to 92, 89, 89, and 80%, respectively, of that at BE. At AE-3, the O_3-inhibition recovered fully in the $EO^{0.1}$ and $HO^{0.1}$ plants. However, the inhibition remained slightly (–4% relative to BE) in the $EO^{0.3}$ plants and substantially (–14% relative to BE) in the $HO^{0.3}$ plants, respectively. The two-way ANOVA (Table 3–2–1) for P_N indicated clear negative effects of O_3 from AE-0 to AE-3. In contrast, the vertical leaf inclination gave positive effects on P_N and mitigated the O_3-inhibition of P_N at AE-1 and AE-3.

The g_s of the $EO^{0.1}$, $HO^{0.1}$, $EO^{0.3}$, and $HO^{0.3}$ plants at AE-0 decreased to 54, 54, 39, and 34%, respectively, of that at BE. Thereafter, the O_3-inhibition of g_s began to recover at AE-1as in the case of P_N. The $EO^{0.1}$, $EO^{0.3}$, and $HO^{0.3}$ plants attained 89, 87, and 83%, respectively, of that at BE. At AE-3, the O_3-inhibition recovered fully in the $EO^{0.1}$ and $HO^{0.1}$ plants. However, the inhibition remained in the $EO^{0.3}$ and $HO^{0.3}$ plants (–17 and –18% of BE) (Fig. 3–2–2B). The two-way ANOVA (Table 3–2–1) for g_s indicated a clear negative effect of O_3 from AE-0 to AE-3.

Effects of O_3 and leaf inclination angle on photosystem II

Under the O_3-free condition, the F_v/F_m and F_q'/F_m' were unaffected by attaching lead weights during the measurement periods (Fig. 3–2–2C, D; HO^0 vs. EO^0). At AE-0, the F_v/F_m of the $HO^{0.3}$ plants decreased to 92% of that at BE, but no significant effect was found in other treatments (Fig. 3–2–2C). The two-way ANOVA (Table 3–1–1) for F_v/F_m indicated that the O_3 had a negative effect at AE-0 and then recovered to the standard level by AE-1 in clean air. The vertical leaf inclination interacted positively with O_3 at AE-0.

The F_q'/F_m' of the $EO^{0.3}$ and $HO^{0.3}$ plants decreased to 78 and 74%, respectively, of that at BE at AE-0. These remained, but were not significantly different from those at BE at AE-1 and AE-3 (Fig. 3–2–2D). Two-way ANOVA (Table 3–1–1) for F_q'/F_m' indicated a negative effect of O_3 at AE-0 and AE-1.

Inclination angle and relative light intensity in different leaf positions

As shown in Fig. 3–2–3, the leaf inclination angle became steeper at upper leaf positions in both measurement stages when compared to that at lower positions. The flag (16^{th}) leaf was arranged to be almost vertical. At the heading stage, the flag and 15^{th} leaves were very erect and received low relative light intensities. The 11^{th} and 10^{th} leaves had a rather horizontal arrangement. Therefore, they received high relative light intensities. The 14^{th} to 12^{th} leaves received low relative light intensities because of the shading by other leaves on the main stem and tillers, regardless of intermediate inclination angles between the high and low positions of leaves.

Ascorbic acid contents in different leaf positions

Because of very similar trends of the total content, AA, DHA, and RDS of ascorbic acid expressed per leaf area, specific leaf weight (leaf dry weight/leaf area) and specific leaf fresh weight (leaf fresh weight/leaf area), values per leaf area are presented in Table 3–2–2. The total ascorbic acid and AA contents per leaf area of the flag leaf were higher than those of the lower leaves. The DHA contents per leaf area were not significantly different irrespective of the leaf position. The RDS of ascorbic acid in the flag leaf was slightly higher than that in other positioned leaves (not significant at $P \leq 0.05$, but significant at $P \leq 0.10$).

3 – 2 – 4. Discussion

Imai and Kobori (2008) reported that the inhibition of P_N in rice leaves by short exposure to O_3 was explained considerably by decreased g_s, and at a high O_3 concentration, irreversible dysfunction of guard and mesophyll cells occurred. Furthermore, the results of Chapter 3 Section 1 showed that the P_N, PS II and g_s of flag (16^{th}) leaf at the heading stage were inhibited by O_3 treatment but their damage was smaller and their recovery was faster than those in the topmost, fully expanded 8^{th} leaf at the vegetative stage. Therefore, they interpreted that the difference in leaf inclination angle and/or antioxidant content were associated with the difference in inhibition by O_3. In the present experiment, the total ascorbic acid content per leaf area of the flag leaf was

slightly higher than that of the lower (15^{th} – 13^{th}) leaves (Table 3–2–2). However, such small difference was unable to explain the mitigation of inhibition by O_3, although it seemed not meaningless.

The experiment of this section was conducted to examine whether or not the leaf inclination angle was related to the difference in inhibition as the other possibility of the mitigation of O_3-inhibition. Because the leaf inclination of rice becomes steeper at late growth stages (Ito et al., 1973), it must affect the radiation interception of individual leaves, especially when plants were isolated situation such like the present experiment. To date, few studies show that the light intensity on the leaf surface is involved in the response to O_3 of plants. In spring wheat, Mulholland et al. (1997) reported that the O_3-induced inhibition of P_N in the 8^{th} (flag) leaf was lighter than that in the 5^{th} to 7^{th} leaves. They ascribed this difference to the higher contents of active ribulose 1, 5-bisphosphate carboxylase/oxygenase and the absence of shade acclimation of the flag leaf. In addition, in primary leaves of pinto bean (*Phaseolus vulgaris*), Guidi et al. (2000a) observed that the inhibition of P_N and PSII activities by O_3 was more pronounced under higher light intensities (30 – 1000 μmol m^{-2} s^{-1} PPFD). Because abiotic stresses such as high light intensity and O_3 induce the production of reactive oxygen species (e.g., superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical) and cause oxidative stress to plants (Taiz and Zeiger, 2011), the stress-induced damage is more severe if such factors occur concomitantly (Mittler, 2006). Generally, the photoinhibition of photosynthesis is induced under high intensity light. However, if the plant is exposed to some other stress that induces the oxidative one, such inhibition may be induced even at low light intensity. Therefore, the light intensity might be an important factor to determine the sensitivity to O_3 of a plant (Fiscus et al., 2005). Along with more erect leaves, the relative light intensity of the leaf surface decreases. Therefore, the relative light intensity of the flag leaf is lower than that of other leaves during daytime. The damage of leaf photosynthesis in the horizontal leaf (H) plant must be severer than that in the erect leaf (E) plant if the difference in intercepted radiation is involved in the extent of O_3 inhibition.

The P_N of the $O^{0.1}$ and $O^{0.3}$ plants decreased, but damage of the $HO^{0.1}$ and $HO^{0.3}$ plants was severer than that of the E plants (Fig. 3–2–2A). Coincident with the results of Chapter 3 Section 1, the F_v/F_m was unaffected by O_3 in the flag leaf of the $EO^{0.1}$ and $EO^{0.3}$ plants, but that in the $HO^{0.3}$ plants decreased at AE-0. Furthermore, the F_q'/F_m' of the $EO^{0.3}$ or H plants decreased at AE-0, and the value of $HO^{0.3}$ plants was lower than that of the $EO^{0.3}$ plants, although it was not significantly different (Fig. 3–2–2C, D). These results indicated that the damage of PSII incurred by the flag leaf in the H plants was severer than in the E plants (erect flag leaf). Consequently, the damage of carbon fixation reaction in the H plants was also severer, and the recovery was slower than that in the E plants. However, under the same O_3 concentration, the g_s of E and H plants was similarly inhibited at AE-0 (Fig. 3–2–2B). This indicated that the O_3 negatively affected the guard cell function, as reported by Torsethaugen et al. (1999) who clarified that O_3 inhibited the opening of stomata through the guard cell K^+ channel.

As shown in Fig. 3–2–3, the leaf inclination angle and relative light intensity of the flag leaf were lower than that of the lower leaves (15^{th} to 10^{th}). The O_3-induced damage of photosynthesis in the flag leaf of $HO^{0.3}$ plant was severer than that of the $EO^{0.3}$ plant in this section (Fig. 3–2–2). Thus, this section concludes that the leaf inclination angle is a major determinant of the O_3 inhibition in rice leaves. In paddy field conditions, however, individual rice plants are affected by mutual shading during growth and receive considerably different incident light within the canopy (San-oh et al., 2008). Furthermore, the profile of canopy O_3 concentration may be complex. Therefore, to elucidate actual O_3-inhibition of photosynthesis-related processes in rice, especially that growing in urban fringe areas, further studies under canopy levels are required.

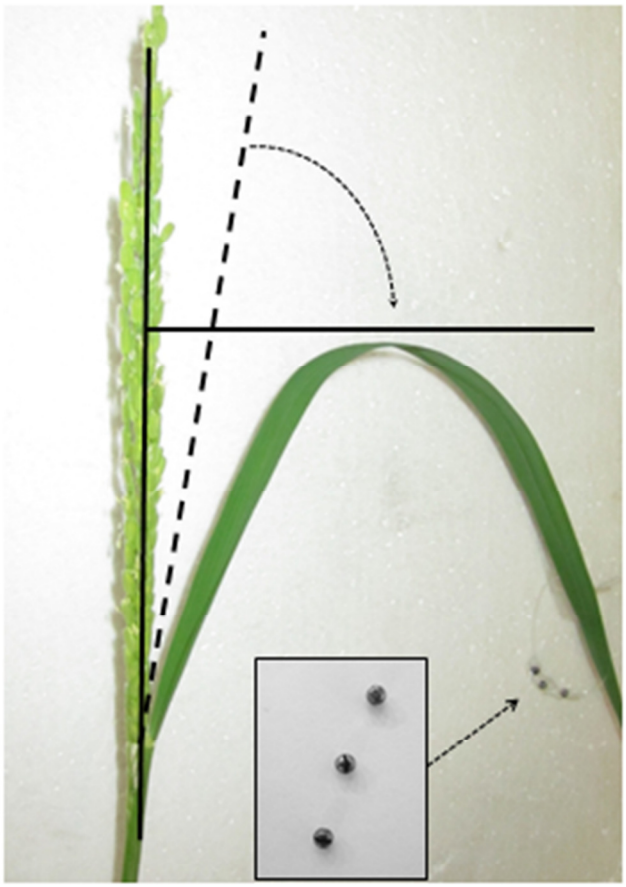


Fig. 3–2–1. Horizontally arranged flag leaf at the middle portion of leaf blade by attaching small, globular lead weights to the tip side. Solid lines show positions of main stem (vertical axis) and horizontally arranged leaves. Dashed line shows the original position of non-treated leaf.

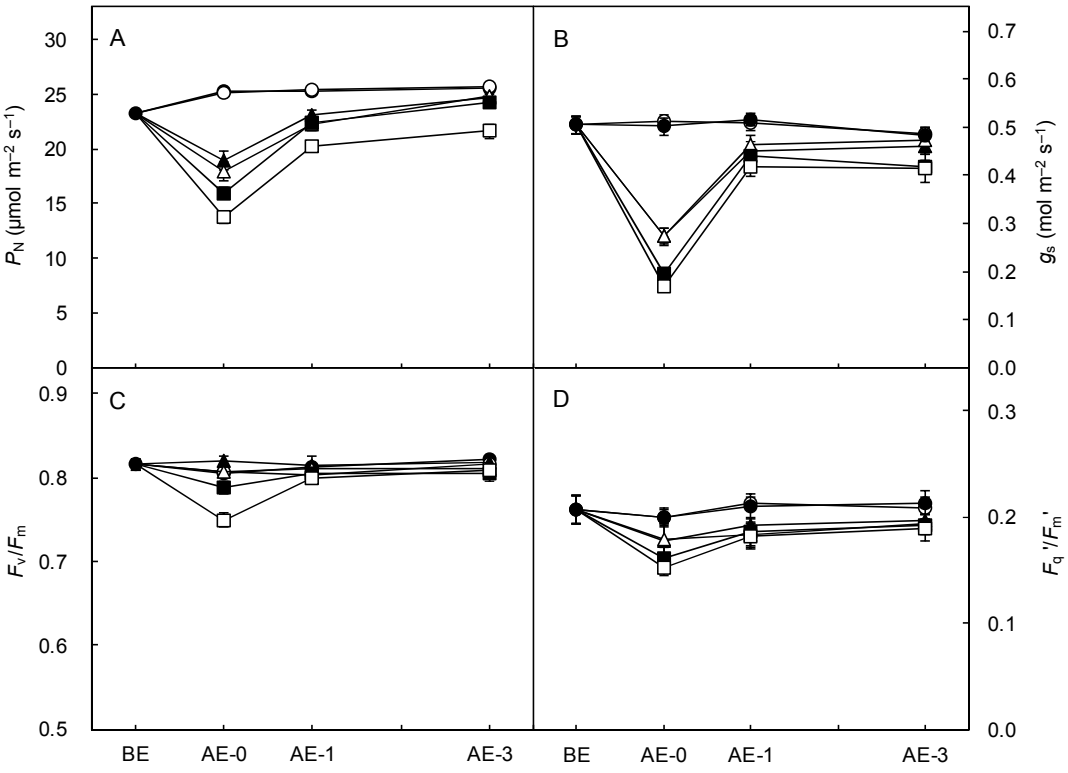


Fig. 3–2–2. Effects of O_3 and inclination angle on the net photosynthesis rate (P_N), stomatal conductance (g_s), maximum quantum efficiency (F_v/F_m) and operating efficiency (F_q'/F_m') of PSII in rice leaves. Vertical bars represent standard error of the means (n = 5). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, and 1d and 3 d after gas exposure. ●, EO^0; ○, HO^0; ▲, $EO^{0.1}$; △, $HO^{0.1}$; ■, $EO^{0.3}$; □, $HO^{0.3}$.

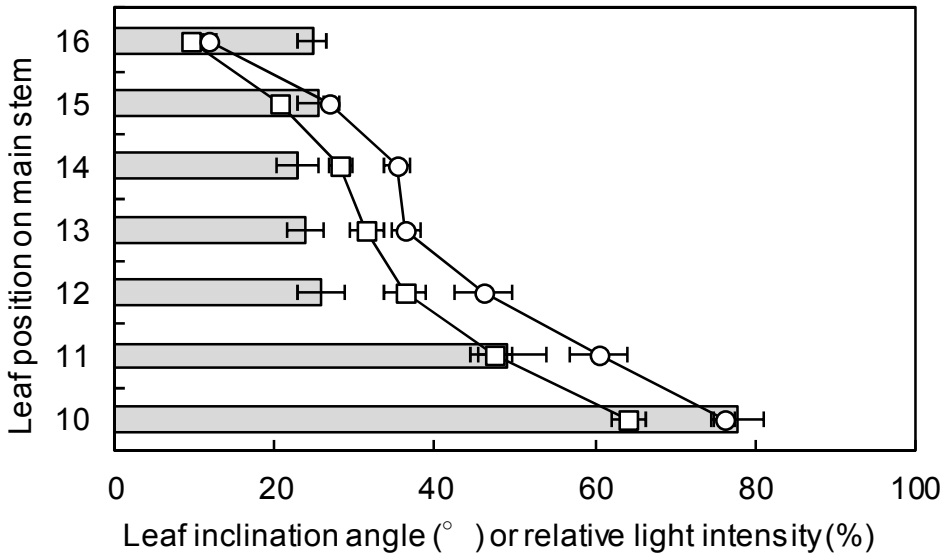


Fig. 3–2–3. The inclination angle and relative light intensity in different leaf positions of rice plant at the heading stage. Bars represent standard errors of the mean (n = 5). ○, inclination angle at the middle portion of the leaf blade; □, inclination angle at the bottom of the leaf blade; bar graph, relative light intensity at the middle of the leaf blade.

Table 3–2–1. Results of statistical analyses of the effects of O_3 and leaf inclination angle on net photosynthetic rate (P_N), stomatal conductance (g_s) and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies of PSII in the flag leaf of rice. AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, and 1 d, and 3 d after gas exposure. *$P \leq 0.05$, **$P \leq 0.01$, n.s. – not significant, by two-way ANOVA.

Time	Factor	P_N	g_s	F_v/F_m	F_q'/F_m'
AE-0	O_3	**	**	**	**
	Leaf inclination	n.s.	n.s.	**	n.s.
	O_3 × Leaf inclination	n.s.	n.s.	*	n.s.
AE-1	O_3	**	**	n.s.	*
	Leaf inclination	**	n.s.	n.s.	n.s.
	O_3 × Leaf inclination	*	n.s.	n.s.	n.s.
AE-3	O_3	**	*	n.s.	n.s.
	Leaf inclination	*	n.s.	n.s.	n.s.
	O_3 × Leaf inclination	**	n.s.	n.s.	n.s.

Table 3–2–2. Content (mmol m^{-2}) of total, reduced form (AA) and oxidized form (DHA) of ascorbic acid and its redox state (RDS) in the 16^{th} (flag leaf) – 13^{th} leaves. In each column, means (n = 4) followed by the same letter are not significantly different at $P \leq 0.05$ level as inferred from results of Tukey's test.

	Leaf position	Total	AA	DHA	RDS
per leaf area	16^{th} (flag leaf)	1.38a	1.03a	0.36a	0.742a
[mmol m^{-2}]	15^{th}	1.17b	0.77b	0.39a	0.671a
	14^{th}	1.15b	0.63b	0.52a	0.550a
	13^{th}	1.14b	0.65b	0.48a	0.580a

Section 3

Effect of Exogenous Ascorbic Acid on O_3-Inhibition of Photosynthesis in Paddy Rice

3 – 3 – 1. Introduction

The results of Chapter 3 Sections 1 and 2 showed that the difference of O_3-inhibition of photosynthesis was ascribable to levels of antioxidants and inclination angle. In this section, the author examined the effect of exogenous ascorbic acid on O_3-inhibition of photosynthesis in paddy rice to clarify the relationship between O_3-inhibition of photosynthesis and antioxidants levels in leaves.

3 – 3 – 2. Materials and methods

Plant materials and gas exposure

On April, japonica rice (*Oryza sativa* L. cv. Koshihikari) seeds were sown directly in Wagner pots (1/5000 a) filled with Kimura B solution (Baba and Takahashi, 1956) with minor element (pH 5.5) (Table. 3–3–1). The nutrient solutions were changed every four days. The plants were cultivated in natural-light gas-exposure chambers (S-2003A; Koito Industries, Yokohama, Japan) as shown in Chapter 2. Immediately after full expansion of the 8^{th} leaves (Haun index = 8.0; Haun, 1973), plants were filled with Kimura B solution including L(+)-ascorbic acid (Kanto Chemical Co., Inc., Japan) [0 (A^0), 5 (A^5), and 10 (A^{10}) mM]. The ascorbic acid treatments induced decrease of pH (pH 3.31-3.70) in nutrient solution. Therefore, the low pH plants (L plants) were prepared to clarify the effect of low pH on the measurement parameters. One day after ascorbic acid treatment, 5 h exposure of O_3 (08:00–13:00; local time) was applied under C^{400}. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 3 Section 1.

Gas-exchange measurements

Using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA),

gas-exchange in the middle portion of flag leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 1.

Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8^{th} leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Ascorbic acid measurements

Four 8^{th} 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 (FW) and leaf area, the leaves were frozen in liquid N_2 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) as shown in Chapter 3 Section 1.

Statistical analysis

Statistical analyses were conducted as shown in Chapter 3 Section 1.

3 – 3 – 3. Results

Effect of Ascorbic Acid on O_3-inhibition of Photosynthesis

Photosynthesis-related parameters in the O^0 plants were unaffected by ascorbic acid application and low pH treatment during AE-0 to AE-3 (Fig. 3–3–1). At AE-0, P_N in the $O^{0.1} + C^{400} + A^0$ and $O^{0.3} + C^{400} + A^0$ plants were respectively 72% and 56% of those at BE. Thereafter, the plants recovered from O_3-inhibition. At AE-3, it had recovered fully in $O^{0.1} + C^{400} + A^0$ plants but

persisted in the $O^{0.3}$ + C^{400} + A^0 plants (–19% of BE). At AE-0, the O_3-inhibition of P_N in the $O^{0.1}$ + C^{400} + A^0 plants showed amelioration by ascorbic acid. At AE-1, P_N in the $O^{0.1}$ + C^{400} + A^5 and $O^{0.1}$ + C^{400} + A^{10} plants had recovered fully. However, the O_3-inhibition of P_N in the $O^{0.3}$ + C^{400} + A^0 plants was unaffected by ascorbic acid during AE-0 to AE-3 (Fig. 3–3–1A). At AE-0, g_s in the $O^{0.1}$ + C^{400} + A^0 and $O^{0.3}$ + C^{400} + A^0 plants were respectively 72% and 56% of those at BE. Thereafter, the plants recovered from O_3-inhibition. At AE-3, it had recovered fully in $O^{0.1}$ + C^{400} + A^0 plants but persisted in the $O^{0.3}$ + C^{400} + A^0 plants (–17% of BE). At AE-0, the O_3-inhibition of g_s in the $O^{0.1}$ + C^{400} + A^0 plants showed amelioration by ascorbic acid; At AE-0, g_s in the $O^{0.1}$ + C^{400} + A^{10} plants was 63% of that at BE. However, the O_3-inhibition of g_s in the $O^{0.3}$ + C^{400} + A^0 plants was unaffected by ascorbic acid during AE-0 to AE-3 (Fig. 3–3–1B). Two-way ANOVA for P_N and g_s showed clear negative effects of O_3 from AE-0 to AE-3 (Table 3–3–2).

At AE-0, the F_v/F_m in the $O^{0.1}$ + C^{400} + A^0 and $O^{0.3}$ + C^{400} + A^0 plants were respectively 91% and 86% of those at BE. Thereafter, the plants recovered from O_3-inhibition. At AE-1, it had recovered fully in $O^{0.1}$ + C^{400} + A^0 plants but persisted in the $O^{0.3}$ + C^{400} + A^0 plants (–5% of BE). At AE-3, the all plants recovered fully from O_3-inhibition. At AE-0, the O_3-inhibition of F_v/F_m in the $O^{0.1}$ + C^{400} + A^0 plants showed amelioration by ascorbic acid. At AE-0, F_v/F_m in the $O^{0.1}$ + C^{400} + A^{10} plants was no affected by O_3. However, the O_3-inhibition of F_v/F_m in the $O^{0.3}$ + C^{400} + A^0 plants was unaffected by ascorbic acid during AE-0 to AE-3 (Fig. 3–3–1C). At AE-0, F_q'/F_m' in the $O^{0.1}$ + C^{400} + A^0 and $O^{0.3}$ + C^{400} + A^0 plants were respectively 78% and 67% of those at BE. Thereafter, the plants recovered from O_3-inhibition. The O_3-inhibition of F_q'/F_m' in the $O^{0.1}$ + C^{400} + A^0 plants showed amelioration by ascorbic acid. At AE-1, F_q'/F_m' in the $O^{0.1}$ + C^{400} + A^5 and $O^{0.1}$ + C^{400} + A^{10} plants were no affected by O_3 (Fig. 3–3–1D). Two-way ANOVA for F_v/F_m and F_q'/F_m' showed clear negative effects of O_3 from AE-0 to AE-3. Because the $O^{0.3}$-inhibition of photosynthesis was not ameliorated by ascorbic acid, there were no interactive effects of O_3 and ascorbic acid during AE-0 to AE-3 (Table 3–3–2).

Effects of O_3 and Exogenous Ascorbic Acid on Endogenous Ascorbic Acid Contents

Total ascorbic acid and RDS of ascorbic acid in the O^0 plants were unaffected by low pH treatment (data not shown). At AE-0, total ascorbic acid was not affected by O_3 and ascorbic acid application. Thereafter, total ascorbic acid in $O^{0.3} + C^{400} + A^0$ plants decreased by O_3, and increased by ascorbic acid application with time. At AE-1 and AE-3, total ascorbic acid in $O^{0.3} + C^{400} + A^0$ plants were respectively 70% and 71% of those at BE. In addition, total ascorbic acid in $O^0 + C^{400} + A^{10}$, $O^{0.1} + C^{400} + A^5$, $O^{0.1} + C^{400} + A^{10}$, $O^{0.3} + C^{400} + A^5$ and $O^{0.3} + C^{400} + A^{10}$ plants at AE-3 were 135%, 148%, 158%, 144% and 155% of those at BE (Fig. 3–3–2A). At AE-0, the RDS of ascorbic acid in the $O^{0.3} + C^{400} + A^0$ plants decreased to 89% of that at BE. At AE-1, the RDS of ascorbic acid further decreased; the RDS of ascorbic acid in $O^{0.1} + C^{400} + A^0$ and $O^{0.3} + C^{400} + A^0$ plants were respectively 87% and 83% of those at BE. Thereafter, the plants recovered from O_3-inhibition. At AE-3, it had recovered fully in $O^{0.1} + C^{400} + A^0$ plants but persisted in the $O^{0.3} + C^{400} + A^0$ plants (–14% of BE). The O_3-inhibition of RDS in the $O^{0.1} + C^{400} + A^0$ and $O^{0.3} + C^{400} + A^0$ plants showed amelioration by ascorbic acid. The decrease of RDS in the $O^{0.1} + C^{400} + A^0$ plants disappeared by ascorbic acid application. In contrast, the decrease of RDS in the $O^{0.3} + C^{400} + A^0$ plants were no affected by ascorbic acid application at AE-0 and AE-1, but at AE-3, the RDS in the $O^{0.3} + C^{400} + A^{10}$ plants were no affected by O_3 (Fig. 3–3–2B). Two-way ANOVA for total ascorbic acid showed clear positive effects of ascorbic acid application at AE-1 and AE-3 ($P \leq 0.001$, Table 3–3–1).

3 – 3 – 4. Discussion

Coincident with a previous study of rice (Imai and Kobori, 2008) and previous section (Chapter 3 Section 1), the photosynthesis-related parameters and content of ascorbic acid and its RDS were inhibited by O_3 exposure (Figs. 3–3–1 and 2). The O_3-inhibition of photosynthesis showed amelioration by ascorbic acid application in the $O^{0.1} + C^{400} + A^0$ plants as reported by Xie et al. (2009), but that in the $O^{0.3} + C^{400} + A^0$ plants was no affected by ascorbic acid application.

Therefore, ascorbic acid is major determination factor for O_3 injury, but cannot fully explain the O_3 injury. In addition, although the total ascorbic acid contents were increased by exogenous ascorbic acid in $O^{0.3}$ + C^{400} + A^5 and A^{10} plants at AE-1 and AE-3, the O_3-inhibiton of photosynthesis-related parameters in $O^{0.3}$ + C^{400} + A^5 and A^{10} plants was no affected by exogenous ascorbic acid. These results showed that the damage of high concentration of O_3 ($O^{0.3}$) may be irreversible damage in rice plants.

Ascorbic acid is an important antioxidant in plant tissue that is synthesized in cytosol of higher plants primarily from conversion of D-glucose to ascorbic acid. Ascorbic acid has been shown to have an essential role in several physiological processes in plants, including growth, differentiation, and metabolism. It functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress. In fact, it reported that exogenous ascorbic acid ameliorated stress damage such as salt (Hasanuzzaman et al., 2013) and high temperature (Shah et al., 2011). In O_3-exposed rice leaves, total ascorbic acid contents were increased by chronic exposure (Nouchi, 1993) and decreased by acute exposure (Frei et al., 2010, Chapter 3 Section 1). Frei et al. (2012) showed the rice mutant ND6172 (ascorbic acid biosynthesis-related enzyme deficient mutant) was more sensitive to O_3 than wild type (Nipponbare) as reported in *Arabidopsis* (Conklin et al., 2000). These reports and results of this section conclude that ascorbic acid is major determination factor for O_3 injury in rice plants as shown in *Arabidopsis*.

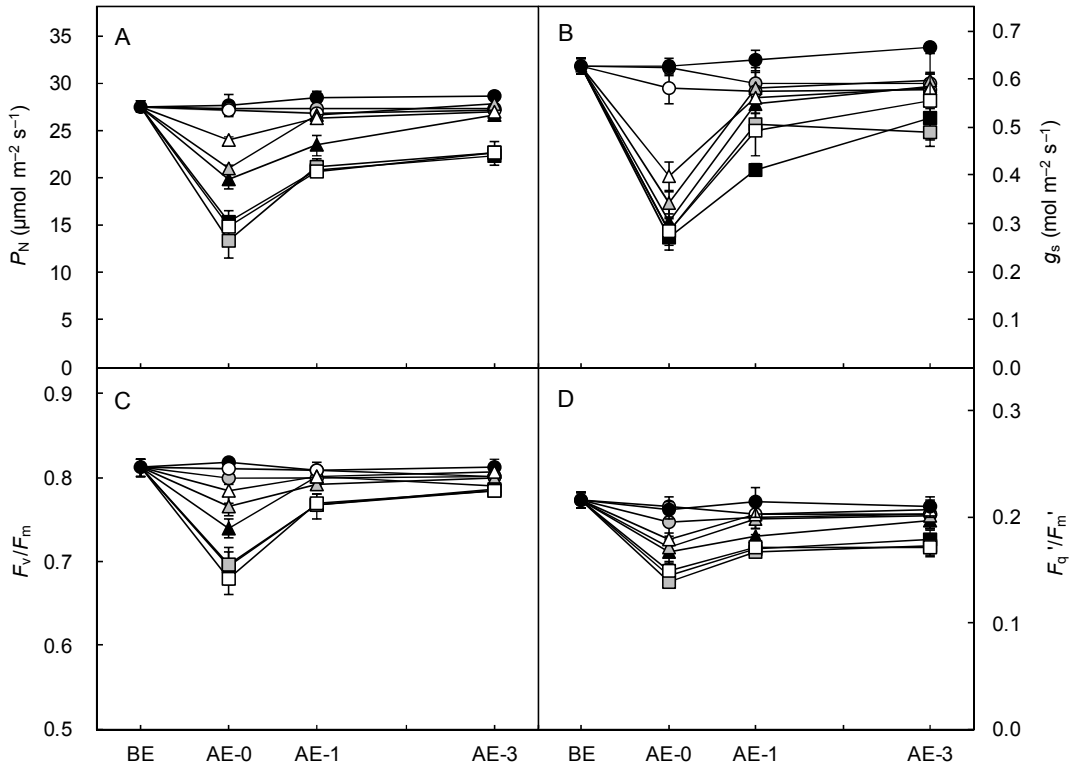


Fig. 3–3–1. Effects of O_3 and ascorbic acid on net photosynthesis rate (P_N), stomatal conductance (g_s), maximum quantum efficiency (F_v/F_m) and operating efficiency (F_q'/F_m') of PSII in rice leaves. Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, and 1 d and 3 d after gas exposure. ●, $O^0 + A^0$; ●, $O^0 + A^5$; ○, $O^0 + A^{10}$; ▲, $O^{0.1} + A^0$; ▲, $O^{0.1} + A^5$; △, $O^{0.1} + A^{10}$; ■, $O^{0.3} + A^0$; ■, $O^{0.3} + A^5$;□, $O^{0.3} + A^{10}$.

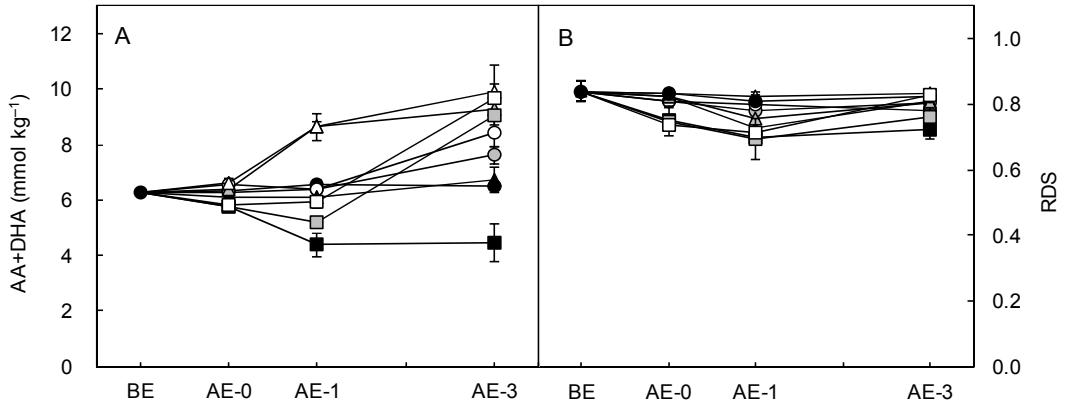


Fig. 3–3–2. Effects of O_3 and ascorbic acid on total ascorbic acid contents and redox state of ascorbic acid (RDS) in rice leaves. Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, and 1 d and 3 d after gas exposure. ●, $O^0 + A^0$; ●, $O^0 + A^5$; ○, $O^0 + A^{10}$; ▲, $O^{0.1} + A^0$; ▲, $O^{0.1} + A^5$; △, $O^{0.1} + A^{10}$; ■, $O^{0.3} + A^0$; ■, $O^{0.3} + A^5$;□, $O^{0.3} + A^{10}$.

Table 3–3–1. Comparision of Kimura B solution (Baba and Takahashi, 1956) with minor element.

Kimura B solution		Minor element	
Comparision	amount (mg/L)	Comparision	amount (mg/L)
$(NH_4)_2SO_4$	48.2 mg/L	H_3BO_3	2.858 mg/L
$MgSO_4$	65.9 mg/L	$MnSO_4 \cdot 5H_2O$	3.601 mg/L
KNO_3	18.5 mg/L	$ZnSO_4 \cdot 7H_2O$	0.879 mg/L
KH_2PO_4	24.8 mg/L	$Na_2MoO_4 \cdot 2H_2O$	0.504 mg/L
$Ca(NO_3)_2$	59.9 mg/L	$CuSO_4 \cdot 5H_2O$	0.786 mg/L
Fe-EDTA	40.4 mg/L		

Table 3–3–2. Statistical analyses of the effects of O_3 and ascorbic acid on photosynthetic parameters and ascorbic acid in rice leaves shown in Figs. 3–3–1 and 2. AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, 1 d, and 3 d after gas exposure. *P < 00.5, ***P < 0.001, n.s. = not significant, as inferred using two-way ANOVA.

Time	Factor	P_N	g_s	F_v/F_m	F'_q/F'_m	AA+DHA	RDS
AE-0	O_3	***	***	***	***	*	***
	AsA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	O_3×AsA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AE-1	O_3	***	***	***	***	***	**
	AsA	n.s.	n.s.	n.s.	n.s.	***	n.s.
	O_3×AsA	n.s.	n.s.	n.s.	n.s.	**	n.s.
AE-3	O_3	***	*	*	**	n.s.	*
	AsA	n.s.	n.s.	n.s.	n.s.	***	n.s.
	O_3×AsA	n.s.	n.s.	n.s.	n.s.	n.s.	*

Section 4

Effects of Acute O_3 Exposure on Leaf Photosynthesis of Paddy Rice Grown at Elevated CO_2 Concentration

3 – 4 – 1. Introduction

Currently, the atmospheric CO_2 concentration is about 391 cm^3 m^{-3}. Its yearly increment over the past decade was about 2 cm^3 m^{-3} (WMO WDCGG, 2013). Therefore, substantially high CO_2 concentrations can be reasonably predicted for the future that 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. In this section, the author examined the effect of acute exposure to O_3 on leaf photosynthesis of paddy rice grown under different CO_2 concentration. In addition, the author 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* (Overmeyer 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 change because of elevated CO_2 in soybean (Pritchard et al., 2000) and forage crops (di Toppi et al., 2002).

3 – 4 – 2. Materials and methods

Plant materials and gas exposure

On April, japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown directly in Wagner pots (1/5000a) as shown in Chapter 2, and plants were cultivated in natural-light gas-exposure chambers (S-2003A; Koito Industries, Yokohama, Japan) at 28/23 °C (12-h day/12-h night), 60% RH and 400 or 800 cm^3 m^{-3} CO_2 (C^{400} or C^{800}) without O_3. Immediately after full expansion of the

8^{th} leaves (Haun index = 8.0; Haun, 1973), 5 h exposure of O_3 (08:00–13:00; local time) 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 $C^{400} + O^0$. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 2. After 5 h gas exposure, the plants were kept at each growth CO_2 concentration without O_3.

Gas-exchange measurements

Using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA), gas-exchange in the middle portion of 8^{th} leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 1 under growth CO_2 concentrations. To clarify the acclimation of P_N and g_s to elevated CO_2 (i.e. down-regulation of them due to long-term growth at elevated CO_2), gas exchange in plants grown in C^{800} and C^{400} conditions was measured at C^{800}. Then the acclimation effect was calculated using the method described by Bunce (2001) as the ratio of P_N (or g_s) measured at C^{800} of the C^{800}-grown plant to that of the C^{400}-grown plant.

Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8^{th} leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Ascorbic acid measurements

Immediately before gas exposure, five fully-unrolled 8^{th} leaves developed at C^{400} and C^{800} were sampled and used for measurements of ascorbic acid (reduced form, AA; oxidized form, DHA). Immediately after measurements of fresh weight (FW) and leaf area, leaves were frozen in liquid

N_2 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) as shown in Chapter 3 Section 1. Using a separate set of plants, the area of the 8th leaf was measured. Its dry weight was obtained after oven-drying at 80 °C for 48 h.

Statistical analysis

Statistical analyses were conducted as shown in Chapter 3 Section 1.

3 – 4 – 3. Results

Acclimation of photosynthesis parameters to elevated CO_2

The P_N and g_s in the $C^{400} + O^0$ and $C^{800} + O^0$ plants immediately before O_3 exposure (BE) are shown in Table 3–4–1. Responses of P_N and g_s of the $C^{800} + O^0$ plants were lower than those of the $C^{400} + O^0$ plants under both CO_2 concentrations measured (C^{400} and C^{800}). Therefore, the acclimatory effect of elevated CO_2 during growth on P_N and g_s was calculated respectively as 0.91 and 0.73. Clear direct effects of elevated CO_2 on P_N (+28 to +29%) and g_s (–12 to –14%) were found but no interaction was found between the CO_2 concentration during plant growth and gas exchange measurements.

Effects of O_3 exposure on photosynthesis in rice grown at ambient or elevated CO_2

At AE-0, the P_N of the $C^{400} + O^{0.1}$ and $C^{400} + O^{0.3}$ plants respectively decreased to 64% and 34% of those at BE (Fig. 3–4–1A). At AE-3, the O_3-inhibition recovered fully in the $C^{400} + O^{0.1}$ plants but remained in the $C^{400} + O^{0.3}$ plants (71% of BE) (Fig. 3–4–1A). In addition, the g_s of the $C^{400} + O^{0.1}$ and $C^{400} + O^{0.3}$ plants at AE-0 respectively decreased to 41% and 22% of those at BE, but they recovered by AE-3 (Fig. 3–4–1B). In $C^{400} \rightarrow C^{800} + O^{0.3}$ 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 $C^{400} + O^{0.3}$ plants (Fig. 3–4–1A, B).

The F_v/F_m of the $C^{400} + O^{0.1}$ and $C^{400} + O^{0.3}$ plants at AE-0 respectively decreased to 91% and 78% of those at BE. At AE-1, the $C^{400} + O^{0.1}$ plants recovered completely, but the $C^{400} + O^{0.3}$ plants showed an inhibition at AE-3, although the difference from the control plants was not significant (Fig. 3–4–1E, Table 3–4–2). The F_q'/F_m' of the $C^{400} + O^{0.1}$ and $C^{400} + O^{0.3}$ plants at AE-0 respectively decreased to 85% and 77% of those at BE. At AE-1, the $C^{400} + O^{0.1}$ plants recovered but the $C^{400} + O^{0.3}$ plants remained low at AE-3 (83% of BE), although the difference from the control plants was not significant (Fig. 3–4–1F, Table 3–4–2). The O_3-inhibition of the F_v/F_m and F_q'/F_m' in the $C^{400} \rightarrow C^{800} + O^{0.3}$ plants at AE-0 was less than that in the $C^{400} + O^{0.3}$ plants (87% and 82% of BE, respectively). At AE-1, the statistical difference between the $C^{400} \rightarrow C^{800} + O^{0.3}$ plants and the control plants diminished (Fig. 3–4–1E, F; Table 3–4–2).

The P_N in the $C^{800} + O^{0.1}$ plants was unaffected by O_3. At AE-0, the P_N of the $C^{800} + O^{0.3}$ plants decreased to 53% of that at BE. The P_N recovered respectively to 72% and 88% at AE-1 and AE-3 (Fig. 3–4–1C). The g_s of the $C^{800} + O^{0.1}$ and $C^{800} + O^{0.3}$ plants at AE-0 respectively decreased to 73% and 61% of that at BE. At AE-1, it recovered in the $C^{800} + O^{0.1}$ plants, but it remained low in $C^{800} + O^{0.3}$ plants (72% of BE) (Fig. 3–4–1D).

The F_v/F_m in the $C^{800} + O^{0.1}$ plants was unaffected by O_3, but that in the $C^{800} + O^{0.3}$ plant at AE-0 tended to decrease (86% of BE). However, it was not significantly different from the control plant at AE-1 and AE-3 (Fig. 3–4–1G, Table 3–4–2). The F_q'/F_m' in the $C^{800} + O^{0.1}$ plants was unaffected by gas exposure, but that in the $C^{800} + O^{0.3}$ plant at AE-0 tended to decrease (84% of BE). It was not significantly different from the control plant at AE-1 and AE-3 (Fig. 3–4–1H, Table 3–4–2).

Results show that there are significant effects of O_3, CO_2, or $O_3 \times CO_2$ 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 3–4–2).

Effect of growth CO_2 concentration on ascorbic acid content

The contents of total ascorbic acid, AA and DHA, and RDS of the 8th 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 weight bases were decreased by C^{800} because the specific leaf weight (SLW), which reflected the leaf thickness, was increased significantly ($P \leq 0.05$) by C^{800}: 3.9 and 4.5 g m^{-2} at C^{400} and C^{800}, respectively (Tables 3–4–2 and 3).

3 – 4 – 4. Discussion

Consistent with the experiments described above, this result 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 CO_2-grown (C^{400}) plants, but the inhibition was smaller in elevated CO_2-grown (C^{800}) plants (Fig. 3–4–1).

Because g_s (i.e. stomatal aperture) was a dominant factor for the O_3-induced inhibition of P_N, the elevated CO_2-grown plants should be less inhibited than ambient CO_2-grown plants because, in this section, the g_s of plants showed clear acclimation (i.e. lower stomatal aperture) to C^{800} (Table 3–4–1). However, the amelioration of O_3-inhibition by C^{800} in the $C^{800} + O^{0.3}$ and $C^{400} \rightarrow C^{800} + O^{0.3}$ plants was similar (Fig. 3–4–1A, C). Consequently, the limitation of O_3 uptake through stomata cannot fully explain the O_3-induced inhibition of P_N and its amelioration by elevated CO_2, as shown by the P_N-C_i relations measured under different O_3 and CO_2 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 CO_2 to the restriction of O_3 uptake through stomata has been inferred for spring wheat (Mulholland et al., 1997). However, causal factor(s) of the amelioration by elevated CO_2 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 O_3 (0.2 cm^3 m^{-3}) exposure of soybean plants grown at elevated CO_2 (650 cm^3 m^{-3}) finding that the sensitivity of plants to acute O_3 exposure was increased because of decreased antioxidant capacity. In relation to this, they observed unchanged total phenolic content that might relate to scavenge O_3 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 CO_2 (350, 700, and 1,050 cm^3 m^{-3}). Ghasemzadeh and Jaafar (2011) grew ginger plants at ambient and elevated CO_2 (400 vs. 800 cm^3 m^{-3}) and observed increased levels of total flavonoids, total phenolics, and antioxidant activity at elevated CO_2. In addition, Pritchard et al. (2000) grew two soybean genotypes (resistant vs. susceptible to frogeye leaf spot disease) at ambient and elevated CO_2 (365 vs. 720 cm^3 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 CO_2. However, those of dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase were increased in susceptible genotype but were decreased in tolerant genotype at elevated CO_2. Schwanz and Polle (2001) reported that elevated CO_2 (700 and 1,200 cm^3 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 CO_2 (ambient vs. 560 cm^3 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 CO_2. Therefore, it is difficult to draw a general scheme of the antioxidative responses of plants to elevated CO_2, probably because of species and/or genotype specific reasons. Regarding responses of plants to elevated CO_2 and O_3, Gillespie et al. (2011) claimed that these two factors differently affected the antioxidant system.

To clarify whether ascorbic acid is actually effective to scavenge O_3-stress in rice leaves or not, this section examined the effect of long-term CO_2 enrichment on ascorbic acid according to some discrepancies (Nouchi, 1993; Imai and Kobori, 2008). Table 3–4–3 shows that elevated CO_2 concentration during growth did not affect the synthesis of ascorbic acid, but its apparent content

decreased when expressed on fresh and dry weight bases because of increased SLW. However, although the RDS of ascorbic acid was not significantly different, the leaves developed at C^{800} showed slightly lower values than that at C^{400}, suggesting that the reactive oxygen species (ROS) was increased somewhat by elevated CO_2. Cheeseman (2006) reported that elevated CO_2 (550 cm^3 m^{-3}) increased the H_2O_2 level in soybean leaves by 42% compared to that at ambient CO_2 (375 cm^3 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 CO_2. Because no such information exists for rice plants, we must examine whether the ROS was increased or not at elevated CO_2, as well as the phenolics.

In summary, the photosynthesis parameters of elevated CO_2-grown rice plants were inhibited by acute O_3 exposure, but the inhibition was less than that of ambient CO_2-grown plants when compared at each growth CO_2 concentration. However, the ascorbic acid content was almost unchanged during the growth at elevated CO_2. Therefore, it did not explain the amelioration by elevated CO_2.

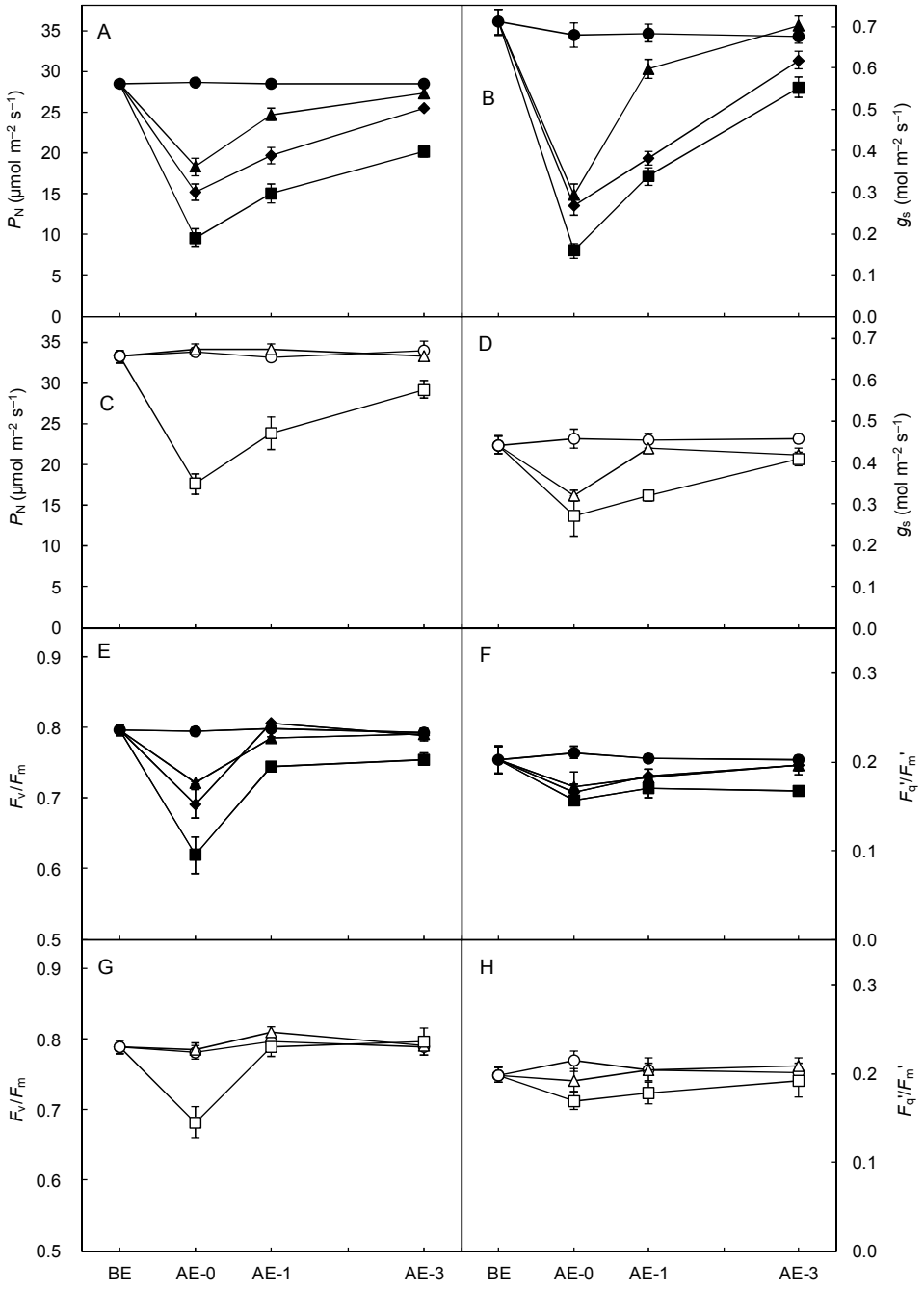


Fig. 3–4–1. Effects of acute O_3 exposure on net photosynthetic rate (P_N), stomatal conductance (g_s), maximum efficiency (F_v/F_m) and operating efficiency (F_q'/F_m') of PSII in rice plants growing at ambient (C^{400}) or elevated (C^{800}) CO_2 concentration. Vertical bars represent standard errors of the means (n = 5). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after O_3 exposure. ●, $C^{400} + O^0$; ○, $C^{800} + O^0$; ▲, $C^{400} + O^{0.1}$; △, $C^{800} + O^{0.1}$; ■, $C^{400} + O^{0.3}$; □, $C^{800} + O^{0.3}$; ♦, $C^{400} \rightarrow C^{800} + O^{0.3}$.

Table 3–4–1. Effects of increased CO_2 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 $cm^3\ m^{-3}$ CO_2 in the $O^0 + C^{800}$ plant to that in the $O^0 + C^{400}$ plant. ***$P \leq 0.001$, n.s. = not significant, as inferred using two-way ANOVA.

Growth CO_2	Measured CO_2	P_N	g_s	Acclimatory effect	
[$cm^3\ m^{-3}$]	[$cm^3\ m^{-3}$]	($\mu mol\ m^{-2}\ s^{-1}$)	($mol\ m^{-2}\ s^{-1}$)	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}	***	***		
	$G_{CO2} \times M_{CO2}$	n.s.	n.s.		

Table 3–4–2. Statistical analyses of the effects of O_3 and CO_2 on gas exchange and chlorophyll fluorescence parameters in rice leaves shown in Fig. 3–4–1. AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, 1 d, and 3 d after gas exposure. *$P \leq$ 0.05, ***$P \leq$ 0.001, n.s. = not significant, as inferred using two-way ANOVA.

Time	Factor	P_N	g_s	F_v/F_m	F'_q/F'_m
AE-0	O_3	***	***	***	***
	CO_2	***	n.s.	*	n.s.
	$O_3 \times CO_2$	***	***	*	n.s.
AE-1	O_3	***	***	***	*
	CO_2	***	***	***	n.s.
	$O_3 \times CO_2$	n.s.	***	n.s.	n.s.
AE-3	O_3	***	***	n.s.	n.s.
	CO_2	***	***	n.s.	n.s.
	$O_3 \times CO_2$	n.s.	***	n.s.	n.s.

Table 3–4–3. Effects of growth CO_2 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 $P \leq 0.05$ level as inferred from results of one-way ANOVA.

	CO_2 [$cm^3\ m^{-3}$]	Total	AA	DHA	RDS
Per fresh weight	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 weight	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

Chapter 4

Effects of O_3 and CO_2 on Nitrate Reduction in Leaves of Paddy Rice

4 – 1. Introduction

As explained above, the photosystem in photosynthesis process is affected by O_3. Therefore, its related metabolism, which receives a reducing power from NADH or ferredoxin (Fd), might also be affected by O_3. Nitrogen metabolism is particularly important for plant growth because it regulates photosynthesis and the C/N ratio. As the key enzyme of nitrogen metabolism in leaves, nitrate reductase (NR) catalyzes the conversion from NO_3^- to NO_2^- using NADH and nitrite reductase (NiR), from NO_2^- to NH_4^+ using Fd (Buchanan et al., 2000; Meyer and Stitt, 2001; Taiz and Zeiger, 2010).

The objective of this chapter is to examine the effects of acute, combined exposure of O_3 and CO_2 on PSII, NR, and NiR.

4 – 2. Materials and methods

Plant materials and gas exposure

On August, japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown directly in Wagner pots (1/5000a), and plants were cultivated in a natural-light greenhouse as shown in Chapter 3 Section 1. Just after the full expansion of 7th leaf (Haun index = 7.0; Haun, 1973), the plants were transferred into natural-light gas-exposure chambers (S-2003A; Koito Industries, Ltd., Yokohama, Japan) and kept at 28/23°C (12–h day/12–h night), 60% RH and 400 cm^3 m^{-3} CO_2 (O^0 + C^{400}). Immediately after full expansion of the eighth leaf, a 5-h gas exposure treatment (08:00–13:00; local time) was applied under several combinations of O_3 and CO_2: O^0 + C^{400} (control = clean air), O^0 + C^{800}, $O^{0.1}$ or $O^{0.3}$ + C^{400}, and $O^{0.1}$ or $O^{0.3}$ + C^{800}. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 3 Section 1. After the gas exposure treatment, all plants were kept

in the same chamber for 3 d under 28/23°C, 60% RH and 400 cm^3 m^{-3} CO_2 (O^0 + C^{400}).

Chlorophyll fluorescence measurements

Using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8th leaves was measured at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Nitrate reductase and nitrite reductase measurements

Simultaneously with the chlorophyll fluorescence measurement, the 8th leaves were sampled from four replicate plants. Immediately after the measurement of fresh weight (FW), the leaves were frozen in liquid N_2 and freeze-dried with a freeze-dryer (FDU-2200; Tokyo Rikakikai Co. Ltd., Tokyo, Japan). With a slight modification of Maeda et al. (2009), ca. 100 mg samples were ground with a mortar and pestle in liquid N_2 and were then suspended in 300 μL extracting solvent [50 mM HEPES-KOH (pH = 7.5), 5 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM DTT, 0.5 mM PMFS, 5 mg PVP]. The extract was centrifuged at 5200 × g (CHIBITAN II; Nihon Millipore Ltd., Tokyo, Japan) for 10 min. Then a part of supernatant (100 μL) was added to 150 μL reagent for NR measurement (50 mM HEPES-KOH [pH = 7.5], 1 mM DTT, 2 mM KNO_3) and incubated for 1 min at 30°C. Twenty minutes after the addition of 50 μL NADH (60 mM) to the reaction mixture, the reaction was completed by adding 450 μL chilled water with intensive stirring. Another part of the supernatant (50 μL) was added to 200 μL reagent for NiR measurement [50 mM HEPES-KOH (pH = 7.5), 1 mM DTT, 2 mM methyl viologen, 1.5 mM $NaNO_2$] and incubated for 1 min at 30°C. Ten minutes after the addition of 50 μL $Na_2S_2O_4$ (600 mM; dissolved in 0.3 mM $NaHCO_3$) to the reaction mixture, the reaction was completed by adding 450 μL chilled water with intensive stirring. The NR or NiR activity was assayed by adding 2.95 mL coloring reagent (mixture of 2.5 g sulfanilamide, 0.05 g N-[1–naphthyl]ethylenediamine dihydrochloride, 31.25 mL hydrochloric acid (12 N) and 706.25 mL H_2O) to the reaction mixture. Twenty minutes

later, the increase (NR) or decrease (NiR) of NO_2^- [standard reagent: $NaNO_2$ (Wako Pure Chemical Industries Ltd., Tokyo, Japan)] was determined using a spectrophotometer (Ubest-30; Jasco Corp., Tokyo, Japan) at 540 nm.

Statistical analysis

Statistical analyses were conducted as shown in Chapter 3 Section 1.

4 – 3. Results

Effects of O_3 and CO_2 on photosystem II

Fig. 4–1A shows that the F_v/F_m in the $O^{0.1} + C^{400}$, $O^{0.1} + C^{800}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants at AE-0 were 93, 97, 87 and 93%, respectively, of those at BE. Thereafter, the O_3-inhibition of F_v/F_m began to recover at 1 d after gas exposure (AE-1), the $O^{0.1} + C^{800}$ plants recovered completely and the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants attained to 97, 93, and 94%, respectively, of the level at BE. At AE-3, all plants recovered from O_3-inhibition. The two-way ANOVA (Table 4–1) for F_v/F_m indicated clearly that treatment of O_3 and CO_2, respectively, had significant negative and positive effects with interaction between them at AE-0 and then recovered to the standard level by AE-3 in clean air.

As presented in Fig. 4–1B, the F_q'/F_m' in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants at AE-0 was 84, 76, and 83%, respectively, of those at BE. At AE-1, all plants, except $O^{0.3} + C^{400}$ plants, recovered from the O_3-inhibition of F_q'/F_m'. The $O^{0.3} + C^{400}$ plants remained inhibited at AE-1 (79% of BE), but recovered by AE-3. They were not significantly different from control plants. Two-way ANOVA (Table 4–1) for F_q'/F_m' indicated a clear negative effect of O_3 only at AE-0.

Effects of O_3 and CO_2 on nitrate and nitrite reductase activities

The effect of O_3 and CO_2 on NR activity in no plant was significantly different from that of the

control plants ($O^0 + C^{400}$) during the measurement periods, although the $O^{0.3} + C^{800}$ plant showed a slightly low activity (Fig. 4–1C, Table 4–1). However, the values reflecting NiR activity in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$ and $O^{0.3} + C^{800}$ plants were 61, 46, and 61%, respectively, of those at BE. At AE-1, these plants began to recover at AE-1 (69, 65, and 62% of those at BE). By AE-3, they had almost recovered (Fig. 4–1D). The two-way ANOVA (Table 4–1) for NiR indicated that a clear negative effect of O_3 was sustained until AE-1.

4 – 4. Discussion

Coincident with previous chapter, the PSII activity (checked for F_v/F_m and F_q'/F_m') of rice leaves was inhibited by O_3 exposure treatment, but it was ameliorated by the treatment concurrently with elevated CO_2 (Fig. 4–1, Table 4–1). Because the inhibition of the photosystem limits the supply of reducing power in the downstream of photosynthetic electron flow, metabolic systems using Fd and NADH would be inhibited. In such an electron flow, the downstream of Fd includes not only the thioredoxin and ascorbic acid, but also NiR, sulfite reductase (SiR), and glutamate synthase (GOGAT) (Foyer, 2002; Taiz and Zeiger, 2010). Because NiR is as important for nitrogen metabolism as NR is, understanding of the effect of O_3 and CO_2 on NiR activity is necessary for evaluation of the carbon/nitrogen balance in plants.

In studies of nitrogen metabolism of broad bean (*Vicia faba*) and chick pea *(Cicer arietinum*), the NR and NiR activities were suppressed at 20 d and 10 d, respectively, by chronic exposure of O_3 (0.098 cm^3 m^{-3}) (Agrawal and Agrawal, 1990). In addition, in sessile oak (*Quercus petraea*), the NiR activity was suppressed by chronic exposure of O_3 (0.08 cm^3 m^{-3}) (Schmadel-Hagebölling et al., 1998). In contrast, no report of research work describes acute exposure of O_3 on enzymes related to nitrogen metabolism. Therefore, the result of this chapter is the first report of acute exposures of O_3 and CO_2 on the NR and NiR activities in leaves of paddy rice, a staple food crop in Japan. Fig. 4–1 shows that the NiR activity was inhibited by O_3 and ameliorated by elevated CO_2, but the NR activity was unaffected by O_3. The NiR is a plastid

(including chloroplast) enzyme which uses Fd as a reducing power and the NR is a cytoplasmic enzyme which uses NADH as a reducing power in leaves (Buchanan et al., 2000; Meyer and Stitt, 2001; Taiz and Zeiger, 2010). Because the NiR activity was inhibited by O_3 similarly to the cases of F_v/F_m and F_q'/F_m' (Fig. 4–1), these results surmised that the inhibition of photosystem was limited the electron transport toward the downstream of photosystem where Fd is located; then the deficient reducing power dropped NiR activity (Fig. 4–1D). However, it remains impossible to clarify the role of elevated CO_2, which scavenges detrimental effects of O_3, although a substantial contribution of elevated CO_2 was performed through stomatal closure (Booker and Fiscus, 2005; Imai and Kobori, 2008).

After reduction from NO_2^- to NH_4^+ by NiR, the NH_4^+ is converted to glutamine by glutamine synthetase and then to glutamic acid by glutamate synthase (GOGAT) (Buchanan et al., 2000; Hirel and Lea, 2001; Taiz and Zeiger, 2010). As in the case of NiR, the GOGAT uses Fd for reducing power. Therefore, it might be affected by acute O_3 exposure. Because the chloroplast is a major organelle that generates reactive oxygen species (ROS), chloroplast NiR might respond to a greater extent to O_3 exposure than the response of cytoplasmic NR. Furthermore, the decline of NiR activity is inferred to induce NO_2^- accumulation. In this chapter, however, the NiR activity was far greater than that of NR activity (Fig. 4–1). Therefore, the toxicity by surplus accumulation of NO_2^- would not occur in rice leaves.

This chapter showed that a key enzyme of nitrogen metabolism (NiR) received negative effects of acute O_3 exposure. Once the nitrogen metabolism is inhibited, the production of photosynthesis-related nitrogen compounds, i.e. chlorophyll and Rubisco, decrease. Therefore, photosynthesis receives a negative effect from O_3. In contrast, once the photosystem is inhibited, the reducing power is limited and the NiR receives negative effects. Therefore, photosynthesis and nitrogen metabolism are inferred to receive detrimental effects of O_3. In addition, SiR in sulfur metabolism and chloroplast GOGAT might be influenced by O_3 because they use Fd as a reducing power, as in the case of NiR (Buchanan et al., 2000; Taiz and Zeiger, 2010). Furthermore, because

elevated CO_2 singly affect the C/N ratio of plants (Imai, 1988), plants in the future are expected to be affected by the complex effects of O_3 and CO_2.

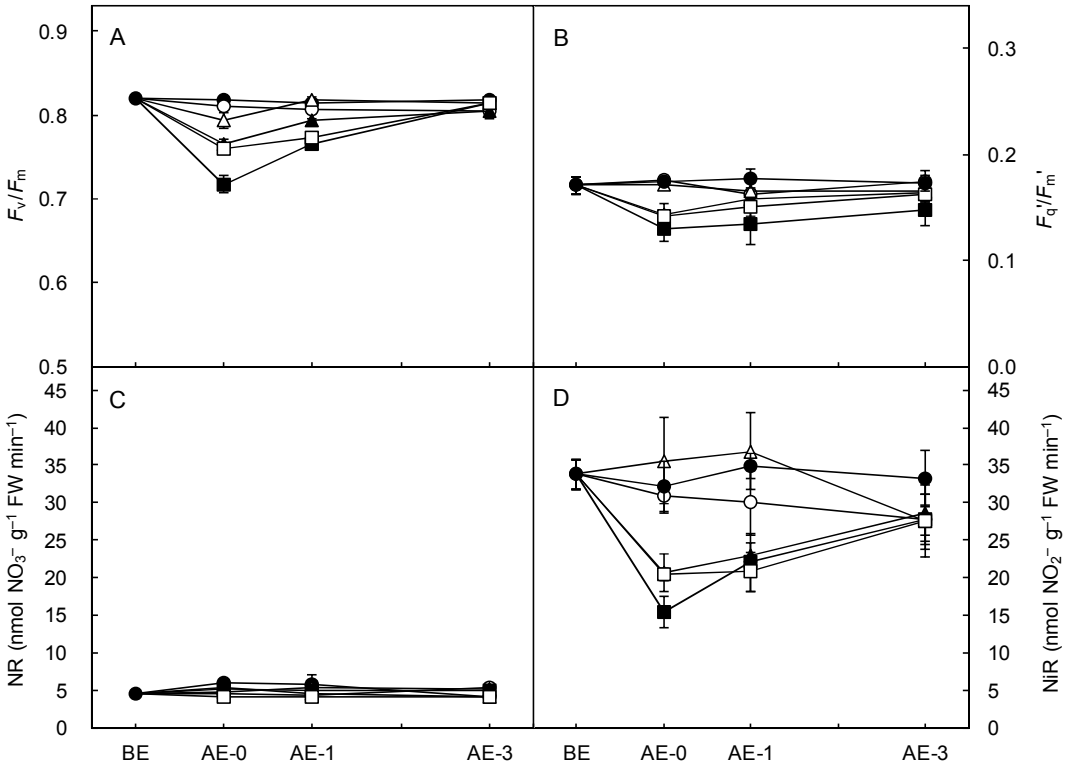


Fig. 4–1. Effects of O_3 and CO_2 on maximum (F_v/F_m), operating (F_q'/F_m') quantum efficiencies of PSII, nitrate (NR) and nitrite (NiR) reductase activities in rice leaves. Vertical bars indicate standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, and 1 d and 3 d after gas exposure. ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

Table 4–1. Results of statistical analyses of the effects of O_3 and/or CO_2 on the maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies, and nitrate (NR) and nitrite (NiR) reductase activities of the eighth leaves of rice shown in Fig. 4–1. AE-0, AE-1, and AE-3 respectively denote values obtained immediately after, 1 d, and 3 d after gas exposure. *$P \leq$ 0.05, **$P \leq$ 0.01, ***$P \leq$ 0.01, n.s. – not significant, by two-way AVOVA.

Time	Factor	F_v/F_m	F'_q/F'_m	NR	NiR
AE-0	O_3	***	***	n.s.	**
	CO_2	**	n.s.	*	n.s.
	$O_3 \times CO_2$	**	n.s.	n.s.	n.s.
AE-1	O_3	***	n.s.	n.s.	*
	CO_2	n.s.	n.s.	n.s.	n.s.
	$O_3 \times CO_2$	**	n.s.	n.s.	n.s.
AE-3	O_3	n.s.	n.s.	n.s.	n.s.
	CO_2	n.s.	n.s.	n.s.	n.s.
	$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.

Chapter 5

Effects of Plant Hormones on O_3-Inhibition of Photosynthetic in Leaves of Paddy Rice

Section 1

Relations between O_3-Inhibition of Photosynthesis and Jasmonic Acid in Paddy Rice Grown under Different CO_2 Concentrations

5 – 1– 1. Introduction

The jasmonates, including jasmonic acid (JA) and methyl jasmonate (MeJA), are related to growth and developmental processes such as seed germination, flower development, leaf senescence, and insect and disease resistance (Creelman and Mullet, 1997). Recently, jasmonates have been shown to function as an important signal in abiotic stresses including O_3 (Rao and Davis, 2001). In *Arabidopsis thaliana* (Rao et al., 2000) and hybrid poplar (Koch et al., 2000), the JA contents were increased by O_3 exposure. Reportedly, JA or MeJA application before O_3 exposure mitigated injury symptoms by O_3 in tobacco (Örvar et al., 1997). Furthermore, because the O_3 injury of plants in JA-insensitive *Arabidopsis* mutant *jar1* (Overmyer et al., 2000) and *oji1* (Kanna et al., 2003) were severer than their wild type, the jasmonates are believed to serve a vital role in ameliorating O_3 injury.

This section described the evaluation of the role of JA in defending against acute O_3-inhibition of P_N in paddy rice grown under different CO_2 concentrations by application of MeJA exogenously and by quantifying endogenous JA and MeJA.

5 – 1– 2. Materials and methods

Plant materials, gas exposure treatments, and MeJA applications

On August (Exp. 1) and June (Exp. 2), japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were

sown directly in Wagner pots (1/5000a) as shown in Chapter 2, and plants were cultivated in natural light growth chambers (width × depth × height = 2.7 m × 3.6 m × 1.8 m, 3S-310; Koito Industries, Ltd., Yokohama, Japan) (Exp. 1) or natural-light gas-exposure chambers (S-2003A; Koito Industries, Ltd., Yokohama, Japan) (Exp. 2) at 28/23 °C (12-h day/12-h night), 70% RH and 400 or 800 cm^3 m^{-3} CO_2 (C^{400} or C^{800}) without O_3. In Exp. 1, immediately after full expansion of the 7th leaves (Haun index = 7.0; Haun, 1973), the plants were transferred to natural-light gas-exposure chambers (S-2003A; Koito Industries, Ltd., Yokohama, Japan) at the same conditions as those described above for growth chambers. Immediately after full expansion of the 8th leaves (Haun index = 8.0), 0.3 mL MeJA (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) solutions [0 (M^0), 10 (M^{10}), and 100 (M^{100}) μM in 0.5% Tween 20 solution] were applied using soft brushes. One day after the MeJA application (Exp. 1) or immediately after full expansion of the 8th leaves (Exp. 2), rice plants were exposed to O_3 (O^0, $O^{0.1}$, and $O^{0.3}$) during 5-h local daytime (08:00–13:00) under growth CO_2 concentrations. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 2.

Gas-exchange measurements

In Exp. 1, using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA), gas-exchange in the middle portion of 8th leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 1 under growth CO_2 concentrations.

Chlorophyll fluorescence measurements

In both Exp. 1 and Exp. 2, using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8th leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Ascorbic acid measurements

In Exp. 1, four 8th 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 (FW) and leaf area, the leaves were frozen in liquid N_2 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) as shown in Chapter 3 Section 1.

Jasmonates (JA and MeJA) measurements

In Exp. 2, four 8th leaves for each treatment were sampled and used for measurements of jasmonates simultaneously with chlorophyll fluorescence measurements. The jasmonates contents were determined as described by Kondo et al. (2000) with a slight modification. Immediately after measurements of the FW, leaf sample (ca. 3 g FW) was homogenized in 20 mL diethyl ether (including 0.005 % butylhydroxytoluene), 10 mL saturated sodium chloride and 0.5 mL 1M citric acid using homogenizer (T10 Basic + S10N-10G; IKA®-Werke Gmbh & Co. KG, Staufen, Germany). The extract was centrifuged at 2000 × g (CF16RX; Hitachi Koki Co, Ltd., Tokyo, Japan) for 15 min at 0 °C. The ether layer was dried at 50 °C using a pressured gas blowing concentrator (MG-2200 + S-024; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) with N_2. The sample was dissolved into the solution of 200 μL chloroform/N, N-diisopropylethylamine, 1:1 (v/v) and 10 μL pentafluorobenzyl bromide, and was derivatized for 60 min at 50 °C. Then, the solution was re-evaporated to dryness at 50 °C under gaseous N_2. The dried sample was re-dissolved in 2 mL *n*-hexane and was injected into a silica gel column. The sample absorbed in this silica gel column was dissolved into *n*-hexane/diethyl ether, 2:1 (v/v) and re-evaporated to dryness at 50 °C under gaseous N_2. The dried sample was kept at –80 °C in a deep freezer (ESL-70A; Ebara Inc., Tokyo, Japan) until assay. Thereafter, the dried sample was re-dissolved in 1 mL methanol and

was filtered and removed pigments using DISMIC®-13HP (Toyo Roshi Kaisha Ltd., Tokyo, Japan) and SEP Cartridge CARBOGRAPH (GL Sciences Inc., Tokyo, Japan). Then, the solution was re-evaporated to dryness at 50 °C under gaseous N_2. The dried sample was re-dissolved in 30 μL methanol and was injected into a gas chromatography – mass spectrometer (GCMS-QP2010; Shimadzu Corp., Kyoto, Japan). Quantification was conducted by internal reference method using ibuprofen (internal standard). 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 200 °C and 250 °C. The column temperature was held at 120 °C for 1 min and then elevated by 14 °C min^{-1} to 280 °C. The retention times of acyl derivatives of JA, MeJA and ibuprofen (Fig. 5–1–1) were, respectively, 10.892, 7.192 and 6.975 min. Ion strength for JA was monitored by m/z 390 and 209. For MeJA, it was monitored by m/z 224 and 193, and for ibuprofen, it was monitored by m/z 206 and 161. The JA and MeJA concentration in the original extract was determined from the ratio of peak areas of m/z 390 (JA) / 206 (ibuprofen) and 224 (MeJA) / 206 (ibuprofen), respectively.

Statistical analysis

All data were subjected to a three-way (Exp. 1) or two-way (Exp. 2) analysis of variance (ANOVA) with Excel Statistics 2010 for Windows software package (Social Survey Research Information Co., Tokyo, Japan). The significance among treatments in each time interval was determined by Tukey's test. Appropriate standard errors of the means (SE) were calculated, and the results are presented as line graphs.

5 – 1– 3. Results

Effect of MeJA on O_3-inhibition of photosynthesis at ambient CO_2 (Exp. 1)

At AE-0, the P_N of the $O^{0.1} + C^{400} + M^0$ and $O^{0.3} + C^{400} + M^0$ plants were found to have decreased respectively to 65% and 44% of those at BE (Fig. 5–1–2A). Thereafter, the O_3-inhibition of P_N

showed recovery at AE-1. At AE-3, it recovered almost entirely in the $O^{0.1} + C^{400} + M^0$ plants (–8% of BE) but persisted in the $O^{0.3} + C^{400} + M^0$ plants (–43% of BE). The P_N was also found to have decreased by MeJA application at AE-0: $O^0 + C^{400} + M^{10}$ and $O^0 + C^{400} + M^{100}$ plants decreased respectively to 87% and 81% of those at BE. The MeJA worsened the O_3-inhibition of P_N at AE-0 but it was showed amelioration at AE-1 and AE-3. At AE-3, the P_N of the $O^{0.3} + C^{400} + M^{10}$ and $O^{0.3} + C^{400} + M^{100}$ plants had recovered to 81% and 85% of those at BE. The g_s of the $O^{0.1} + C^{400} + M^0$ and $O^{0.3} + C^{400} + M^0$ plants at AE-0 decreased respectively to 43% and 37% of those at BE (Fig. 5–1–2B). Thereafter, the O_3-inhibiton of g_s recovered somewhat by AE-1, as in the case of P_N. At AE-3, inhibition had recovered fully in the $O^{0.1} + C^{400} + M^0$ plants but persisted in the $O^{0.3} + C^{400} + M^0$ plants (–47% of BE). The MeJA had decreased g_s at AE-0: $O^0 + C^{400} + M^{100}$ plants decreased to 81% of those at BE. As shown in P_N, the MeJA worsened the O_3-inhibition of g_s at AE-0, but ameliorated at AE-1 and AE-3. At AE-3, the g_s of the $O^{0.3} + C^{400} + M^{10}$ and $O^{0.3} + C^{400} + M^{100}$ plants recovered to 80% and 85% of those at BE. The three-way ANOVA (Table 5–1–1) for P_N and g_s indicated a clear negative effect ($P \leq 0.001$) of O_3 from AE-0 to AE-3. At AE-1 and AE-3, interactive effects ($P \leq 0.01$ at AE-1, $P \leq 0.001$ at AE-3) were found between O_3 and MeJA on P_N because MeJA ameliorated O_3-inhibiton of P_N.

The F_v/F_m of the $O^{0.1} + C^{400} + M^0$ and $O^{0.3} + C^{400} + M^0$ plants at AE-0 had decreased respectively to 92% and 87% of those at BE (Fig. 5–1–2C). The O_3-inhibition of F_v/F_m was ameliorated by MeJA: F_v/F_m of the $O^{0.1} + C^{400} + M^{10}$, $O^{0.1} + C^{400} + M^{100}$, $O^{0.3} + C^{400} + M^{10}$, and $O^{0.3} + C^{400} + M^{100}$ plants were 95%, 97%, 94%, and 96% of those at BE. Thereafter, the O_3-inhibition of F_v/F_m recovered fully except in $O^{0.3} + C^{400} + M^0$ plants, which showed continued inhibition at AE-3. The three-way ANOVA (Table 5–1–1) for F_v/F_m revealed a clear negative effect ($P \leq 0.001$) of O_3 from AE-0 to AE-1. At AE-0 and AE-1, an interactive effect ($P \leq 0.001$) was found between O_3 and MeJA on F_v/F_m because MeJA ameliorated the O_3-inhibiton of F_v/F_m.

The F_q'/F_m' of the $O^{0.1} + C^{400} + M^0$ and $O^{0.3} + C^{400} + M^0$ plants at AE-0 had decreased respectively to 81% and 70% of those at BE (Fig. 5–1–2D). MeJA had ameliorated O_3-inhibition

of F_q'/F_m' at AE-0: F_q'/F_m' of the $O^{0.1} + C^{400} + M^{10}$, $O^{0.1} + C^{400} + M^{100}$, $O^{0.3} + C^{400} + M^{10}$, and $O^{0.3} + C^{400} + M^{100}$ plants were 85%, 90%, 82%, and 80% of those at BE. The O_3-inhibition of F_q'/F_m' showed a recovery at AE-1. At AE-3, the inhibition by $O^{0.1}$ had recovered fully. However, the inhibition by $O^{0.3}$ persisted at AE-3 irrespective of the amelioration by MeJA. The respective F_q'/F_m' of the $O^{0.3} + C^{400} + M^0$, $O^{0.3} + C^{400} + M^{10}$, and $O^{0.3} + C^{400} + M^{100}$ plants were 85%, 88%, and 91% of those at BE. Three-way ANOVA (Table 5–1–1) for F_q'/F_m' indicated a clear negative effect ($P \leq 0.001$) of O_3 from AE-0 to AE-3.

Effect of MeJA on O_3-inhibition of photosynthesis at elevated CO_2 (Exp. 1)

The O_3-inhibition of all photosynthesis-related parameters was ameliorated by elevated CO_2 (Fig. 5–1–3A-D). The P_N of the $O^{0.1} + C^{800} + M^0$ plants was unaffected by O_3 from AE-0 to AE-3 (Fig. 5–1–3A). At AE-0, the P_N of the $O^{0.3} + C^{800} + M^0$ plants had decreased to 69% of those at BE, and MeJA had worsened the inhibition by $O^{0.3}$ at AE-0: P_N of the $O^{0.3} + C^{800} + M^{10}$ and $O^{0.3} + C^{800} + M^{100}$ plants decreased respectively to 66% and 60% of those at BE. However, MeJA ameliorated the O_3-inhibition of P_N at AE-1 and AE-3 and at AE-3, the P_N of the $O^{0.3} + C^{800} + M^{100}$ plants recovered fully. At AE-0, the g_s was found to have been decreased by O_3 and MeJA. The g_s of the $O^0 + C^{800} + M^{10}$, $O^0 + C^{800} + M^{100}$, $O^{0.1} + C^{800} + M^0$, $O^{0.1} + C^{800} + M^{10}$, $O^{0.1} + C^{800} + M^{100}$, $O^{0.3} + C^{800} + M^0$, $O^{0.3} + C^{800} + M^{10}$, and $O^{0.3} + C^{800} + M^{100}$ plants at AE-0 had decreased respectively to 83%, 69%, 51%, 59%, 53%, 38%, 40%, and 39% of those at BE (Fig. 5–1–3B). Thereafter, inhibition by O_3 and MeJA began to recover at AE-1. At AE-3, the inhibition had recovered almost entirely, except the $O^{0.1} + C^{800} + M^{100}$, $O^{0.3} + C^{800} + M^0$, and $O^{0.3} + C^{800} + M^{10}$ plants. Three-way ANOVA (Table 5–1–1) for P_N and g_s revealed a clear effect ($P \leq 0.001$) of CO_2 and interactive effect ($P \leq 0.001$) between O_3 and CO_2 from AE-0 to AE-3.

The F_v/F_m of the $O^{0.3} + C^{800} + M^0$ plants at AE-0 had decreased to 91% of those at BE (Fig. 5–1–3C). The O_3-inhibition of F_v/F_m later showed recovery at AE-1 and at AE-3. It recovered fully in all plants. MeJA ameliorated O_3-inhibition of F_v/F_m. At AE-0, the F_v/F_m of the $O^{0.1} + C^{800} + M^{10}$

and $O^{0.1}$ + C^{800} + M^{100} plants were found to be unaffected by O_3. The F_v/F_m of the $O^{0.3}$ + C^{800} + M^{10} and $O^{0.3}$ + C^{800} + M^{100} plants at AE-0 had decreased respectively to 93% and 98% of those at BE. At AE-1, the O_3-inhibition of the $O^{0.3}$ + C^{800} + M^{100} plants had recovered fully. The F_q'/F_m' of the $O^{0.1}$ + C^{800} + M^0 plants was unaffected by O_3 from AE-0 to AE-3 (Fig. 5–1–3D). At AE-0, the F_q'/F_m' in the $O^{0.3}$ + C^{800} + M^0 plants had decreased to 77% of those at BE, but MeJA ameliorated the inhibition. The O_3-inhibition of F_q'/F_m' had recovered fully in the $O^{0.3}$ + C^{800} + M^{100} plants at AE-1 and at AE-3: no significant differences were found among plants. Three-way ANOVA (Table 5–1–1) for F_v/F_m and F_q'/F_m' indicated an interactive effect ($P \leq 0.001$) between O_3 and CO_2 at AE-0.

Effects of O_3 and MeJA on ascorbic acid contents under different CO_2 concentrations (Exp. 1)

Because effects of the O_3 and MeJA on total ascorbic acid, AA and DHA contents expressed per fresh weight were similar to those expressed per leaf area, values presented in Fig. 5–1–4 are those obtained on a fresh weight basis. The only exception was that a significant effect of CO_2 on the total ascorbic acid occurring from AE-0 to AE-3 was found when they were expressed per leaf area, although the effect was not significant when expressed per unit of fresh weight (Table 5–1–1).

At AE-0, total ascorbic acid contents of the $O^{0.3}$ + C^{400} + M^0 plants had decreased to 65% of those at BE (Fig. 5–1–4A). At AE-1, the content had decreased further by O_3: total ascorbic acid contents of the $O^{0.1}$ + C^{400} + M^0 and $O^{0.3}$ + C^{400} + M^0 plants were 76 and 38% of those at BE. However, MeJA ameliorated the O_3-inhibition. Total ascorbic acid contents of the $O^{0.1}$ + C^{400} + M^{10}, $O^{0.1}$ + C^{400} + M^{100}, and $O^{0.3}$ + C^{400} + M^{100} plants were unaffected by O_3 from AE-0 to AE-3. In contrast, MeJA increased total ascorbic acid contents, irrespective of the O_3 exposure. At AE-3, total ascorbic acid contents of O^0 + C^{400} + M^{100} plants were found to have increased to 119% of those at BE. At AE-0, total ascorbic acid contents were found to have been unaffected by O_3 under

elevated CO_2 (C^{800}) (Fig. 5–1–4B). At AE-1 and AE-3, total ascorbic acid contents of $O^{0.3} + C^{800} + M^0$ plants were found to have decreased, but those of $O^{0.1} + C^{800} + M^0$ plants were unaffected by O_3. The MeJA ameliorated the O_3-inhibition of $O^{0.3} + C^{800} + M^0$ plants, as in the case of C^{400}. In addition, total ascorbic acid contents increased by M^{100} but not by M^{10} under $O^{0.3} + C^{800}$. Three-way ANOVA (Table 5–1–1) for total ascorbic acid contents showed a clear negative effect ($P \leq 0.001$) of O_3 and a positive effect ($P \leq 0.001$) of MeJA from AE-0 to AE-3.

At AE-0, the RDS of ascorbic acid of $O^{0.3} + C^{400} + M^0$ plants was found to have decreased to 86% of that at BE (Fig. 5–1–4C). The MeJA ameliorated the O_3-inhibition of $O^{0.3} + C^{400} + M^0$ plants. The RDS of $O^{0.3} + C^{400} + M^{10}$ and $O^{0.3} + C^{400} + M^{100}$ plants were 95% of those at BE. The O_3-inhibition recovered fully in $O^{0.3} + C^{400} + M^{10}$ and $O^{0.3} + C^{400} + M^{100}$ plants but it persisted in $O^{0.3} + C^{400} + M^0$ plants from AE-1 to AE-3. Under the C^{800}, the RDS was unaffected by O_3 exposure at AE-0 (Fig. 5–1–4D). At AE-1, the RDS of $O^{0.3} + C^{800} + M0$ plants decreased to 92% of that at BE. The O_3-inhibition in the $O^{0.3} + C^{800} + M^0$ plants fully recovered by M^{100}. Three-way ANOVA (Table 5–1–1) for the RDS showed a clear negative effect ($P \leq 0.001$) of O_3 and a positive effect ($P \leq 0.01$ at AE-0 and AE-1, $P \leq 0.05$ at AE-3) of CO_2 from AE-0 to AE-3. A clear positive effect ($P \leq 0.001$ at AE-1, $P \leq 0.01$ at AE-3) of MeJA, and an interactive effect ($P \leq 0.001$ at AE-1, $P \leq 0.01$ at AE-3) between O_3 and MeJA were observed at AE-1 and AE-3.

Effects of O_3 and CO_2 on jasmonic 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 89%, 68% and 88% of the values recorded at BE; the F_q'/F_m' in the corresponding plants had decreased respectively to 72%, 64% and 71% of those at BE. Thereafter, the F_v/F_m and F_q'/F_m' recovered from O_3-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 91% and 79% of those at BE (Fig. 5–1–5A, B).

At AE-1, the JA contents were no affected by O_3 and CO_2, but the MeJA contents in the $O^{0.1} +$

C^{400}, $O^{0.3}$ + C^{400}, $O^{0.1}$ + C^{800} and $O^{0.3}$ + C^{800} plants had increased respectively to 414%, 727%, 369% and 752% of those at BE. Thereafter, the MeJA contents decreased with time; the MeJA contents in $O^{0.1}$ + C^{400}, $O^{0.3}$ + C^{400} and $O^{0.3}$ + C^{800} plants were 191%, 238% and 369% of those at BE. In contrast to MeJA, the JA contents increased by O_3 with time; At AE-3, the JA contents in $O^{0.3}$ + C^{400} and $O^{0.3}$ + C^{800} plants had increased respectively to 229% and 300% of those at BE (Fig. 5–1–5C, D). Two-way ANOVA for MeJA content showed a clear effect of O_3 from AE-0 to AE-3 ($P \leq 0.001$). In addition, because the JA content was increased by O_3 from AE-1, two-way ANOVA for JA content showed a clear effect of O_3 at AE-1 ($P \leq 0.01$) and AE-3 ($P \leq 0.001$, Table 5–1–2).

5 – 1– 4. Discussion

Coincident with previous study of rice (Imai and Kobori, 2008) and previous chapter (Chapter 3), the photosynthesis-related parameters and content of ascorbic acid and its RDS were inhibited by O_3 exposure, but they were ameliorated by elevated CO_2 (C^{800}) (Figs. 5–1–1, 2 and 3).

In this section, MeJA ameliorated the O_3 inhibition of PSII (F_v/F_m and F_q'/F_m') at both CO_2 concentrations (C^{400} and C^{800}) (Figs. 5–1–1 and 2). In contrast, MeJA did not ameliorate the O_3-inhibition of P_N at AE-0: P_N was decreased by both O_3 and MeJA. However, the recovery of P_N from AE-1 occurred more rapidly because of MeJA treatment.

In JA-treated barley (Popova et al., 1988) and MeJA-treated *Brassica napus* L. (Rossato et al., 2002), the P_N of unstressed plants decreased as in this section. Popova and Vaklinova (1988) reported that the causal factor of the inhibition of P_N by JA is the inhibition of Rubisco synthesis. However, Suhita et al. (2003) showed that the MeJA induced stomatal closure in *Nicotiana glauca* L. In this section, MeJA decreased g_s (Figs. 5–1–1 and 2), suggesting that the causal factor of the inhibition of P_N was stomatal closure. Because the MeJA-induced stomatal closure limited the O_3 flux through stomata, as in the case of elevated CO_2 (Booker and Fiscus, 2005; Imai and Kobori, 2008), the MeJA is expected to ameliorate O_3-inhibition of P_N.

In contrast to gas exchange parameters, the chlorophyll fluorescence parameters were unaffected by MeJA in this section (Figs. 5–1–1 and 2). However, Jung (2004) reported that MeJA accelerated leaf senescence because MeJA had decreased the F_v/F_m and electron transfer rate (ETR) in *Arabidopsis thaliana* at 7 d after application. Similarly, the section showed that MeJA decreased chlorophyll content (SPAD value) of rice leaves at 7 d after application (unpublished observation). The breakdown of chlorophyll with decreasing photosynthesis-related parameters is an index of leaf senescence (Hörtensteiner and Lee, 2007). Therefore, one must be careful when using jasmonates to ameliorate stress including O_3 at actual field sites.

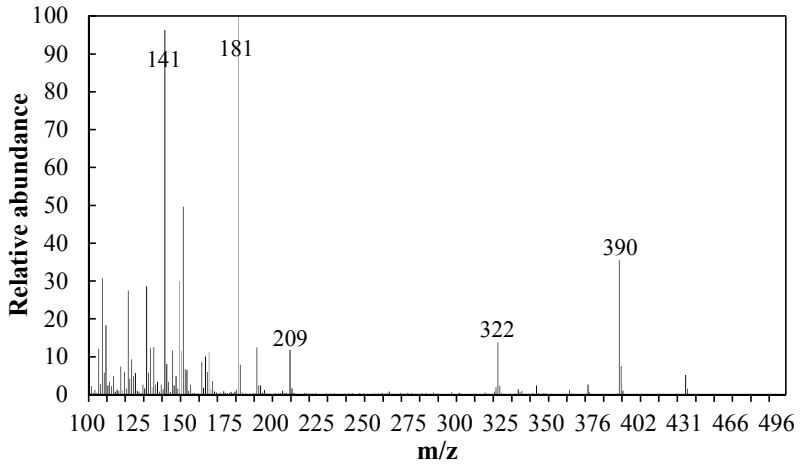
In this section, the total ascorbic acid content was increased, and the O_3-induced decrease of total ascorbic acid content was ameliorated by MeJA (Fig. 5–1–3). Sasaki-Sekimoto et al. (2005) reported that the gene expression of antioxidants (ascorbic acid and glutathione) syntheses and the ascorbate-glutathione cycle-related enzymes were increased by JA or MeJA application in *Arabidopsis thaliana*. Because the MeJA increased phenylalanine ammonia-lyase (PAL) activity in rice (Hung and Kao, 2007), and antioxidative enzymes activities such as superoxide dismutase (SOD) and catalase (CAT) in *Arabidopsis thaliana* (Jung, 2004), the jasmonates (JA and MeJA) are expected to promote the antioxidative and secondary metabolism of plants. As shown in Figs. 5–1–1, 2 and 3, the MeJA played a vital role in the amelioration of O_3-inhibition through the promotion of antioxidative metabolism in rice leaves because its application increased the total ascorbic acid content along with the amelioration of O_3-inhibition. This is coincident with a result of Chapter 3 Section 3 and showed that the ascorbic acid treatment induced the amelioration of O_3-inhibition of P_N, although the amelioration by MeJA was more effective than by ascorbic acid treatment in this section. This result suggests that the promotion of antioxidative metabolism alone is insufficient to ameliorate O_3-inhibition of P_N in rice.

The O_3-inhibition of F_v/F_m and F_q'/F_m' were ameliorated by M^{10} at C^{400} but not ameliorated at C^{800} (Figs. 5–1–1 and 2). Additionally, the respective effects of MeJA on ascorbic acid differed in C^{400} and C^{800} at $O^{0.3}$. The decrease of total ascorbic acid by $O^{0.3}$ was ameliorated by both M^{100} and

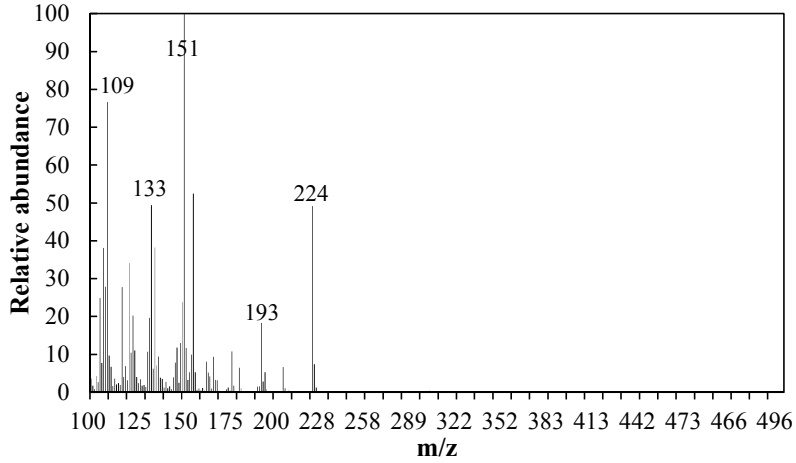
M^{10} at C^{400} but unchanged by M^{10} at C^{800} (Fig. 5–1–3). Suppression of the MeJA effect at C^{800} was probably involved in physiological and morphological factors. The long-term, elevated CO_2 treatment changes plant metabolism in various ways: promotion (di Toppi et al., 2002) or suppression (Gillespie et al., 2011) of antioxidative metabolism, promotion of sugar accumulation (Imai, 1988), promotion of secondary metabolism (Peñuelas et al., 1997; Ghasemzadeh and Jaafar, 2011), and promotion (indoleacetic acid, gibberellin, and cytokinin) or suppression (abscisic acid) of plant hormone synthesis (Teng et al., 2006). These changes might affect the sensitivity of plants to MeJA. However, MeJA uptake through stomata might be suppressed by elevated CO_2 because it induces stomatal closure. Furthermore, the decreased transpiration rate and increased leaf thickness by elevated CO_2 (Imai, 1988) might suppress the uptake and allocation of chemicals from soil. Therefore, one must be cautious about application of chemicals such as JA and MeJA to plants to ameliorate stress including that by O_3 at elevated CO_2 concentration.

Coincident with the reports on *Arabidopsis* (Rao et al., 2000) and hybrid popular (Koch et al., 2000), the O_3 exposure increased the endogenous level of JA and MeJA in rice leaves (Fig. 5–1–5C, D). MeJA contents in rice leaves were increased immediately after O_3 exposure, and decreased from AE-1 to AE-3, although JA contents were increased with a lag time. Therefore, it is predicted that O_3 induces JA biosynthesis, thereafter JA is converted MeJA by jasmonic acid carboxyl methyltransferase (JMT). These results of Chapter 5 Section 1 showed that jasmonates function as positive regulator of O_3-inhibition as case of *Arabidopsis* (Rao and Davis, 2001; Tamaoki, 2008).

A



B



C

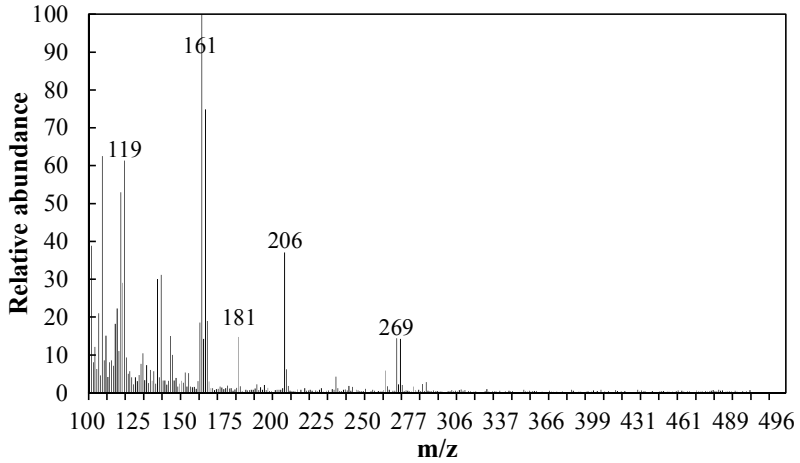


Fig. 5–1–1. Mass spectra of the acyl derivative of jasmonic acid (A), methyl jasmonate (B) and ibuprofen (C).

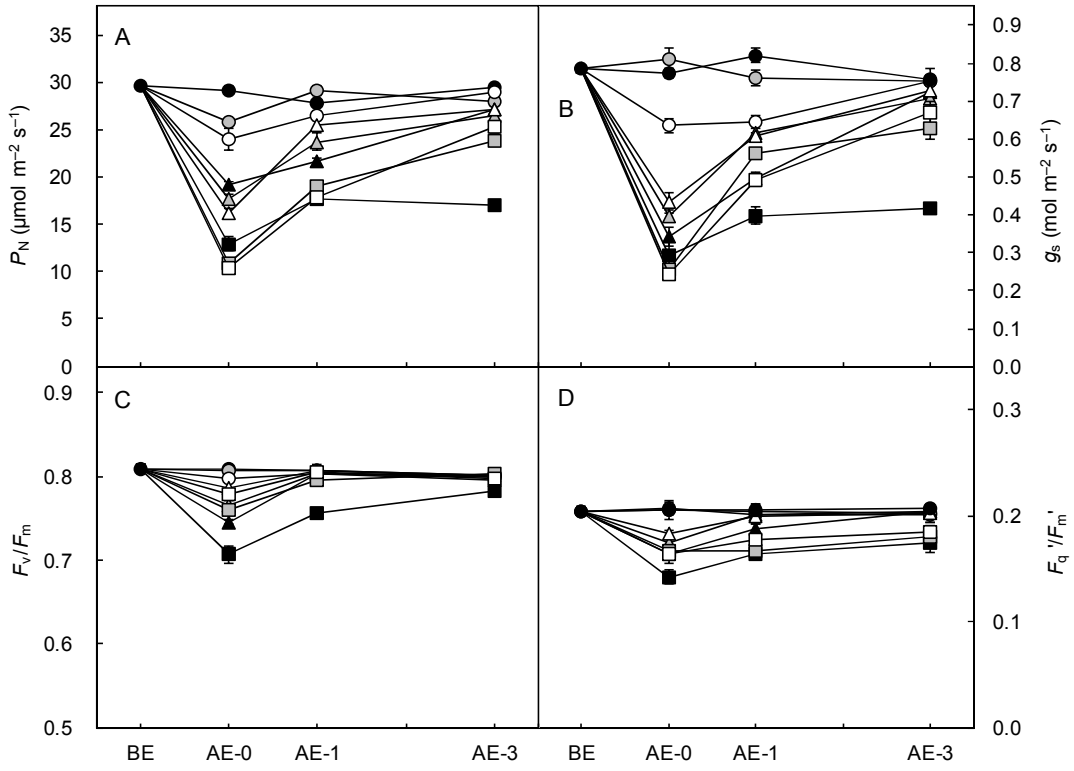


Fig. 5–1–2. Effects of O_3 and methyl jasmonate (MeJA) on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII at ambient CO_2 concentration in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}: 400 cm^3 m^{-3} CO_2; M^0, M^{10}, M^{100}: 0, 10, 100 μM MeJA. ●, $O^0 + C^{400} + M^0$; ●, $O^0 + C^{400} + M^{10}$; ○, $O^0 + C^{400} + M^{100}$; ▲, $O^{0.1} + C^{400} + M^0$; ▲, $O^{0.1} + C^{400} + M^{10}$; △, $O^{0.1} + C^{400} + M^{100}$; ■, $O^{0.3} + C^{400} + M^0$; ■, $O^{0.3} + C^{400} + M^{10}$; □, $O^{0.3} + C^{400} + M^{100}$.

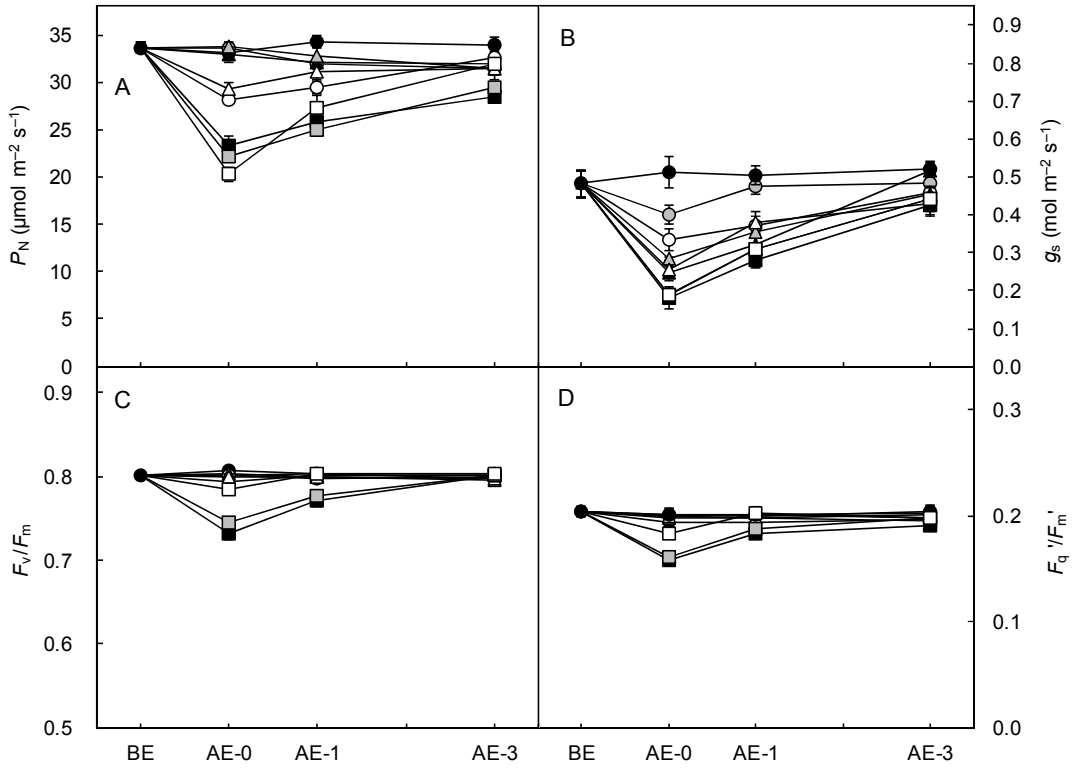


Fig. 5–1–3. Effects of O_3 and MeJA on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII at elevated CO_2 concentration in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{800}: 800 cm^3 m^{-3} CO_2; M^0, M^{10}, M^{100}: 0, 10, 100 μM MeJA. ●, $O^0 + C^{800} + M^0$; ●, $O^0 + C^{800} + M^{10}$; ○, $O^0 + C^{800} + M^{100}$; ▲, $O^{0.1} + C^{800} + M^0$; ▲, $O^{0.1} + C^{800} + M^{10}$; △, $O^{0.1} + C^{800} + M^{100}$; ■, $O^{0.3} + C^{800} + M^0$; ■, $O^{0.3} + C^{800} + M^{10}$; □, $O^{0.3} + C^{800} + M^{100}$.

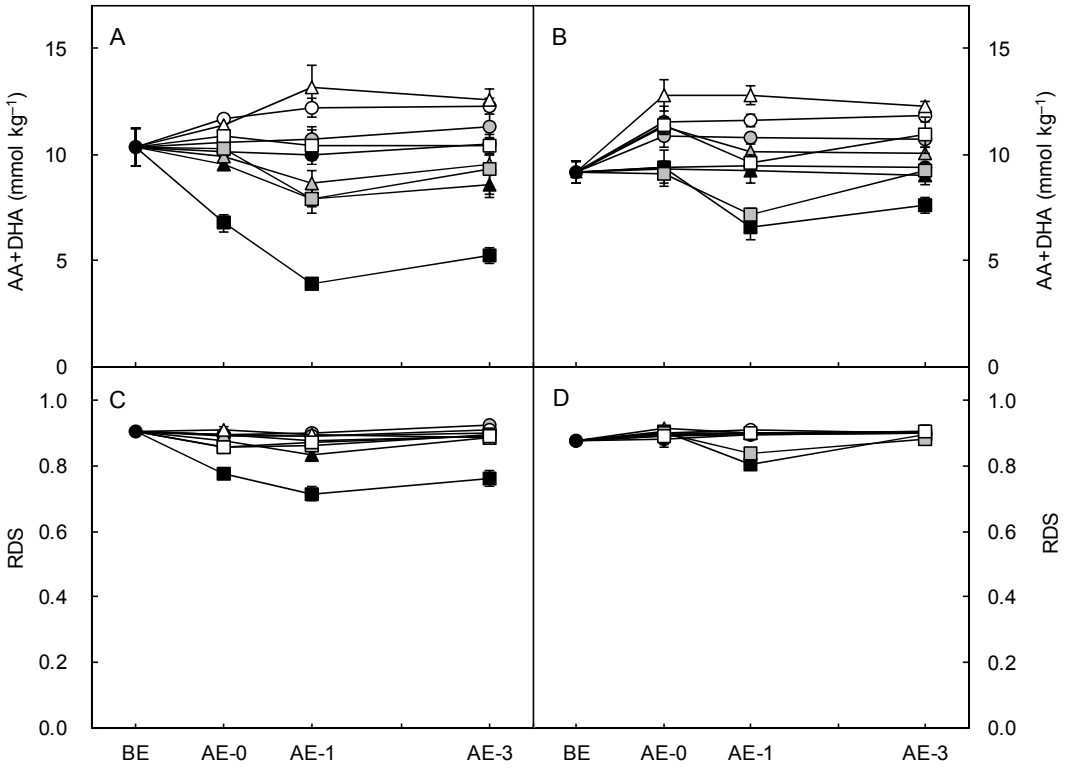


Fig. 5–1–4. Effects of O_3 and MeJA on total ascorbic acid and redox state of ascorbic acid (RDS) at ambient (A, B) and elevated (C, D) CO_2 concentrations in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). AA: reduced form of ascorbic acid; DHA: dehydroascorbic acid (oxidized form of ascorbic acid), RDS: redox state of ascorbic acid [AA/(AA+DHA)]. BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800}: 400, 800 cm^3 m^{-3} CO_2; M^0, M^{10}, M^{100}: 0, 10, 100 μM MeJA. ●, $O^0 + C^{400}$ or $C^{800} + M^0$; ●, $O^0 + C^{400}$ or $C^{800} + M^{10}$; ○, $O^0 + C^{400}$ or $C^{800} + M^{100}$; ▲, $O^{0.1} + C^{400}$ or $C^{800} + M^0$; ▲, $O^{0.1} + C^{400}$ or $C^{800} + M^{10}$; △, $O^{0.1} + C^{400}$ or $C^{800} + M^{100}$; ■, $O^{0.3} + C^{400}$ or $C^{800} + M^0$; ■, $O^{0.3} + C^{400}$ or $C^{800} + M^{10}$; □, $O^{0.3} + C^{400}$ or $C^{800} + M^{100}$.

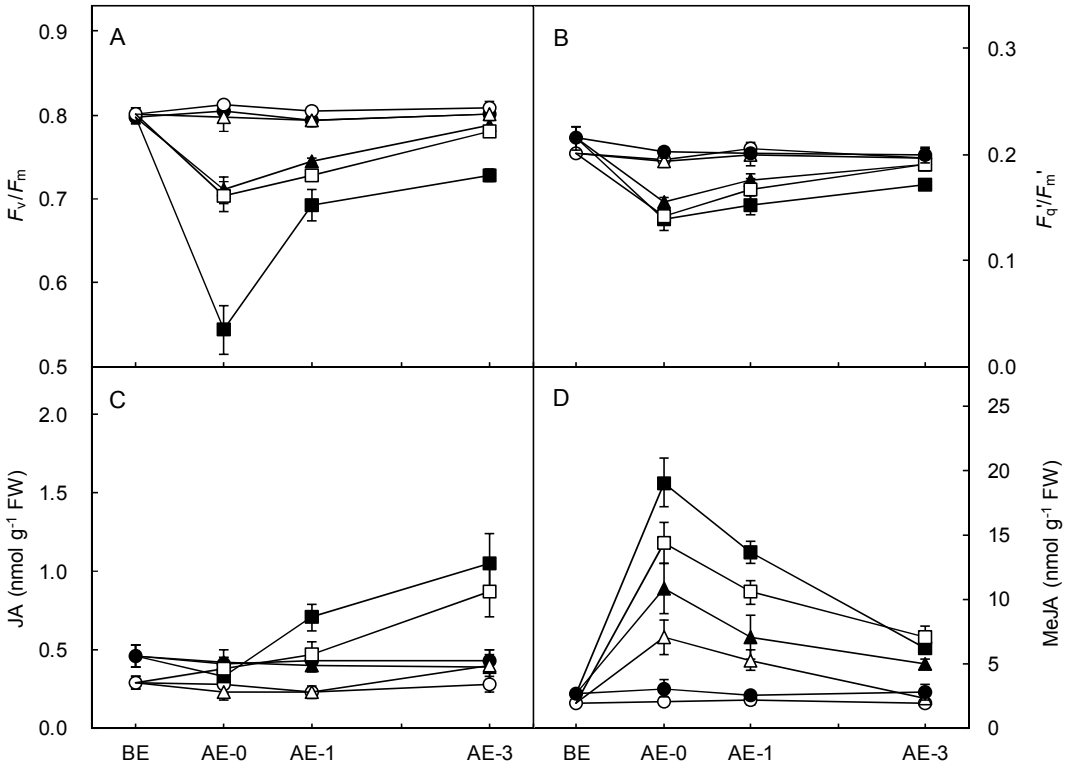


Fig. 5–1–5. Effects of O_3 and CO_2 on maximum (F_v/F_m), operating (F_q'/F_m') quantum efficiency of PSII (A, B), jasmonic acid (JA) and methyl jasmonate (MeJA) contents in rice leaves (Exp. 2). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure; O^0, $O^{0.1}$, $O^{0.3}$ - 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800} - 400, 800 cm^3 m^{-3} CO_2; ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

Table 5–1–1. Statistical analysis of the effects of O_3, CO_2, and MeJA on net photosynthetic rate (P_N), stomatal conductance (g_s), maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII, and total (AA+DHA), reduced (AA), and oxidized (DHA) ascorbic acid contents per fresh weight in rice leaves shown in Figs. 5–1–2, 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	***	***	***	***	***	***	n.s.	***
	CO_2	***	***	***	**	n.s.	*	*	**
	MeJA	***	**	***	**	***	***	n.s.	n.s.
	O_3×CO_2	***	***	***	***	n.s.	n.s.	**	***
	O_3×MeJA	n.s.	***	***	n.s.	n.s.	n.s.	n.s.	n.s.
	CO_2×MeJA	**	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
	O_3×CO_2×MeJA	n.s.	*	*	n.s.	**	**	n.s.	n.s.
AE-1	O_3	***	***	***	***	***	***	n.s.	***
	CO_2	***	***	n.s.	**	n.s.	n.s.	n.s.	**
	MeJA	n.s.	**	**	*	***	***	n.s.	***
	O_3×CO_2	***	***	n.s.	***	n.s.	n.s.	n.s.	n.s.
	O_3×MeJA	**	***	***	n.s.	**	**	n.s.	***
	CO_2×MeJA	*	*	n.s.	n.s.	n.s.	n.s.	n.s.	**
	O_3×CO_2×MeJA	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AE-3	O_3	***	***	n.s.	***	***	***	n.s.	***
	CO_2	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	*
	MeJA	**	n.s.	n.s.	n.s.	***	***	*	**
	O_3×CO_2	***	***	n.s.	*	n.s.	*	n.s.	**
	O_3×MeJA	***	***	n.s.	n.s.	*	*	n.s.	**
	CO_2×MeJA	*	**	n.s.	n.s.	n.s.	n.s.	n.s.	**
	O_3×CO_2×MeJA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**

Table 5–1–2. Statistical analysis of the effects of O_3 and CO_2 on maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII, jasmonic acid (JA) and methyl jasmonate (MeJA) content per fresh weight in rice leaves presented in Fig. 5–1–5 (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'	JA	MeJA
AE-0	O_3	***	***	n.s.	***
	CO_2	***	n.s.	n.s.	*
	$O_3 \times CO_2$	**	*	n.s.	n.s.
AE-1	O_3	***	***	**	***
	CO_2	***	n.s.	**	n.s.
	$O_3 \times CO_2$	n.s.	n.s.	n.s.	n.s.
AE-3	O_3	***	n.s.	***	***
	CO_2	***	n.s.	n.s.	*
	$O_3 \times CO_2$	**	n.s.	n.s.	*

Section 2

Relation between O_3-inhibition of Photosynthesis and Salicylic Acid (SA) in Paddy Rice Grown under Different CO_2 Concentrations

5 – 2– 1. Introduction

Salicylic acid 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 O_3 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 O_3 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 O_3-exposed plants (Kangasjärvi et al., 2005). Consequently, SA is related to the O_3 response of several model plants, although details of its role remain unclear.

This section described the evaluation of the role of SA in defending against acute O_3-inhibition of P_N in paddy rice grown under different CO_2 concentrations by exogenous application of SA and by quantification of endogenous SA.

5 – 2– 2. Materials and methods

Plant materials, gas exposure treatments, and SA applications

On April (Exp. 1) and August (Exp. 2), japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown directly in Wagner pots (1/5000a), and plants were cultivated in a natural-light gas-exposure chamber (S-2003A; Koito Industries, Ltd., Yokohama, Japan) as shown in Chapter 3 Section 4. In Exp. 1, immediately after full expansion of the 8th leaves (Haun index = 8.0; Haun, 1973), 0.3 mL

SA (Wako Pure Chemical Industries Ltd., Oosaka, Japan) solutions [0 (S^0), 0.1 ($S^{0.1}$), and 1 (S^1) mM in 0.5% Tween 20 solution] were applied to the 8th leaves using soft brushes. One day after SA application (Exp. 1) or immediately after full expansion of the 8th leaves (Exp. 2), rice plants were exposed to O_3 (O^0, $O^{0.1}$, and $O^{0.3}$) during 5-h local daytime (08:00–13:00) under growth CO_2 concentrations. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 2.

Gas-exchange measurements

In Exp. 1, using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA), gas-exchange in the middle portion of 8th leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 1 under growth CO_2 concentrations.

Chlorophyll fluorescence measurements

In both Exp. 1 and Exp. 2, using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8th leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Ascorbic acid measurements

In Exp. 1, four 8th 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 (FW) and leaf area, the leaves were frozen in liquid N_2 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) as shown in Chapter 3 Section 1.

Salicylic acid measurements

In Exp. 2, four 8th leaves for each treatment were sampled and used for measurements of SA simultaneously with chlorophyll fluorescence measurements. Immediately after measurements of the FW, leaves were frozen in liquid N_2 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 N_2. 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 N_2. 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. 5–2–1) 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

Statistical analyses were conducted as shown in Chapter 5 Section 1.

5 – 2– 3. Results

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

Photosynthesis-related parameters in the O^0 plants were unaffected by SA application during AE-0 to AE-3 (Fig. 5–2–2). At AE-0, P_N in the $O^{0.1} + C^{400} + S^0$ and $O^{0.3} + C^{400} + S^0$ plants were respectively 54% and 39% of those at BE. Thereafter, the plants recovered from O_3-inhibition. At AE-3, it had recovered substantially in the $O^{0.1} + C^{400} + S^0$ plants (–12% of BE) but persisted in the $O^{0.3} + C^{400} + S^0$ 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. 5–2–2A). 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 $O^{0.1} + C^{400} + S^1$ plants at AE-3 were, respectively, 76% and 77% of the $O^{0.1} + C^{400} + S^0$ plants (Fig. 5–2–2B). 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 5–2–1). 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 5–2–1).

As presented in Fig. 5–2–2C, 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. 5–2–2C). 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. 5–2–2D). 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 5–2–1). 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 5–2–1).

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. 5–2–3). 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. 5–2–3A). 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 recovered fully 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. 5–2–3B). 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 5–2–1).

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 recovered fully from O_3-inhibition. However, it was not ameliorated by SA during AE-0 to AE-1 (Fig. 5–2–3C). 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 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 O_3 in the $O^{0.3}$ + C^{800} + $S^{0.1}$ and $O^{0.3}$ + C^{800} + S^1 plants had disappeared (Fig. 5–2–3D). Three-way ANOVA for F_v/F_m and F_q'/F_m' showed a clear interaction between O_3 and CO_2 at AE-0 ($P \leq 0.001$, Table 5–2–1).

Effects of O_3 and SA on ascorbic acid content under different CO_2 concentrations (Exp. 1)

Because the effects of O_3 and SA on total ascorbic acid, AA and DHA contents expressed per FW were similar to those expressed per leaf area, the values presented in Fig. 5–2–4 are those obtained on a FW 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 O_3-inhibition was less under elevated CO_2 (C^{800}). 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 O_3-inhibition of total ascorbic acid content was ameliorated by SA application. The amelioration became clearer with time (Fig. 5–2–4A, 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 O_3-inhibition of RDS was ameliorated by SA. At AE3, the plants fully recovered from O_3-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 O_3-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 (–20% of BE) and $O^{0.3}$ + C^{400} + $S^{0.1}$ and $O^{0.3}$ + C^{400} + S^1 (–14% of BE) plants (Fig. 5–2–4C, D). Three-way ANOVA for total ascorbic acid and RDS showed clear negative effects of O_3 during AE-0 to AE-3 ($P \leq 0.001$, Table 5–2–1). In addition, because SA ameliorated the negative effect of O_3 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 5–2–1).

Effects of O_3 and CO_2 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 O_3-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. 5–2–5A, B).

The SA contents in the $O^0 + C^{400}$ and $O^0 + C^{800}$ plants were 11–15 μg g^{-1} FW. These are within a range of rice leaves (8–30 μg 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. 5–2–5C). Two-way ANOVA for SA content showed a clear effect of O_3 from AE-1 to AE-3 ($P \leq 0.001$, Table 5–2–2).

5 – 2– 4. Discussion

Salicylic acid 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 (Chapter 5 Section 1), but the amelioration by SA was less than that of MeJA. In addition, photosynthesis-related parameters were unaffected by SA (Figs. 5–2–2 and 3). In contrast to SA, MeJA decreased gas-exchange-related parameters (Popova et al., 1988). The results of Chapter 5 Section 1 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 section, 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.

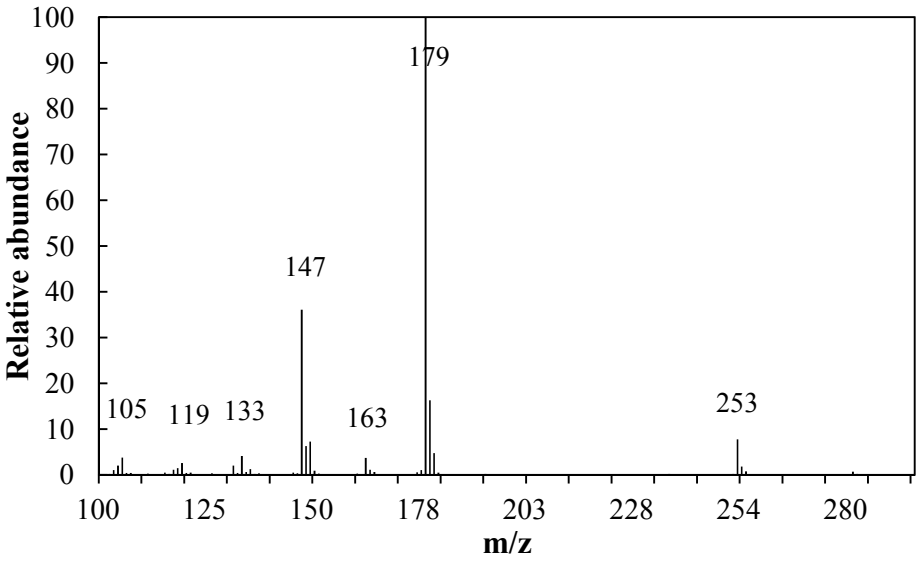
The SA and SA derivatives induce systemic acquired resistance (Oostendorp et al., 2001). Similarly, SA application ameliorates various stresses caused by factors such as heavy metals, salinity, and temperature (Hayat et al., 2010). Zhao et al. (2010) evaluated the effects of exogenously applied SA (0.5–1mM) on chronic O_3 stress in soybean. They showed that O_3-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 O_3 exposure was limited. In *Arabidopsis*, the O_3 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 this section, the O_3-induced inhibition of P_N was ameliorated slightly by SA application (Figs. 5–2–2 and 3). In addition, the decreases in total ascorbic acid and RDS induced by O_3 were ameliorated by SA application, although the total ascorbic acid was unaffected by SA in healthy leaves of the O^0 plants (Fig. 5–2–4). Presumably, SA accelerates the recovery of P_N from O_3 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).

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 O_3 exposure increased the endogenous level of SA in rice leaves (Fig. 5–2–5c). 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 O_3 injury (Kangasjärvi et al., 2005). The results of this section showed that SA content was greater by O_3 at AE-1 and AE-3 than at AE-0. Therefore, SA may be related to the

enlargement of O_3 injury or induction of recovery process, or both. Ogawa et al. (2005a) 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 μg g^{-1} FW) are higher than that of Arabidopsis (0.01–0.1 μg 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 results of this section showed that the effect of SA application is not concentration-dependent (Figs. 5–2–2, 3 and 4). Therefore this section infer that SA does not act as a direct defense component, but instead acts as a signal for inducing defense response in O_3-exposed leaves.

DeLucia et al. (2012) reported that elevated CO_2 stimulated the production of endogenous SA in plants. However, in this section, SA contents were unaffected by the CO_2 concentration (C^{400} vs. C^{800}) in rice leaves. Moreover, DeLucia et al. (2012) showed that elevated CO_2 suppressed the production of endogenous JA. The results of Chapter 5 Section 1 showed that the effect of MeJA application on O_3-inhibition was suppressed slightly by elevated CO_2 (C^{800}). In *Arabidopsis*, syntheses of plant hormones such as indoleacetic acid, gibberellin, and cytokinin are promoted, but abscisic acid is suppressed by elevated CO_2 (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 CO_2 are more susceptible to leaf blast than those grown under ambient CO_2 (Kobayashi et al., 2006). In addition, elevated CO_2-grown soybean plants show a higher sensitivity to acute exposures of O_3 because of the lowered antioxidant capacity under elevated CO_2 (Gillespie et al., 2011).

A



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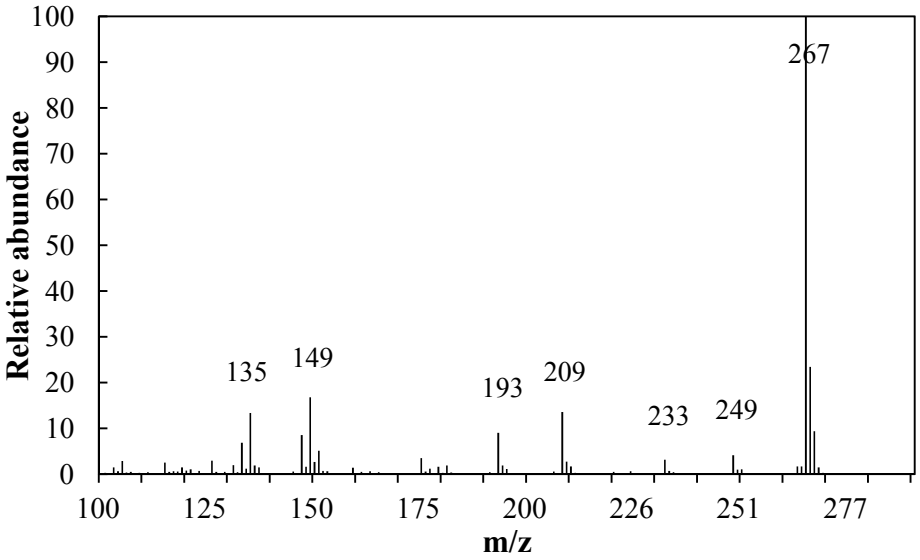

Fig. 5–2–1. Mass spectra of trimethylsilyl derivatives of salicylic acid (A) and mandelic acid (B).

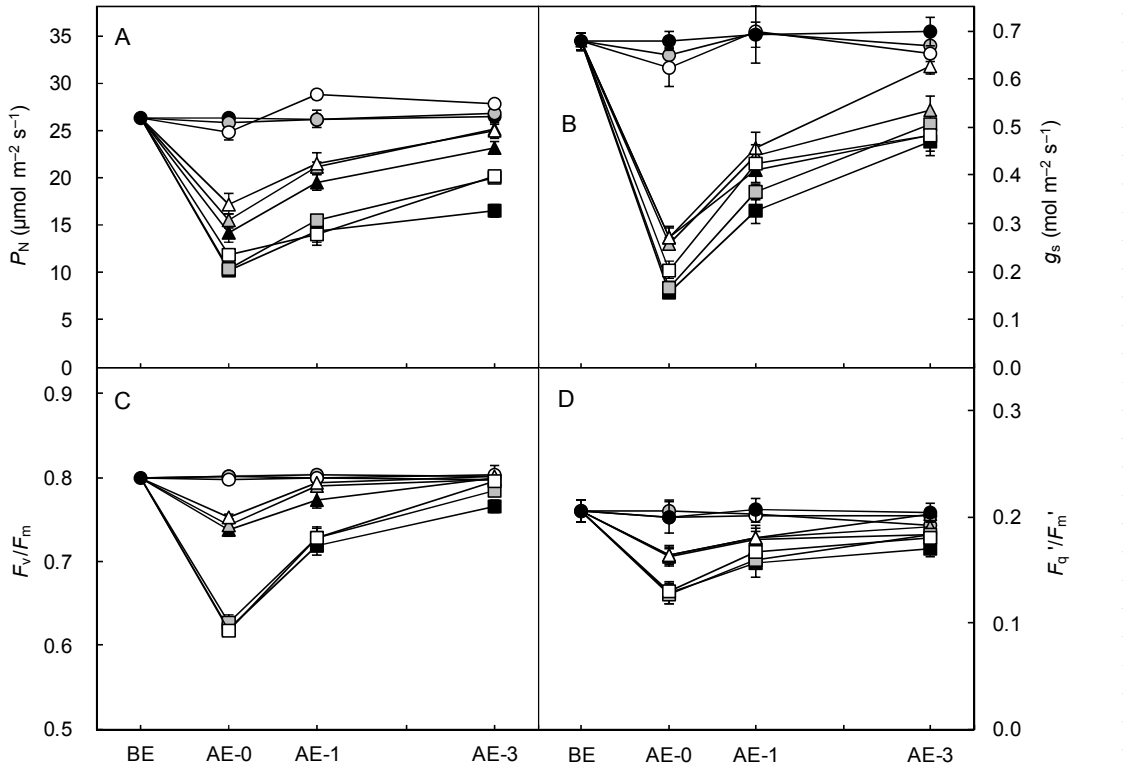

Fig. 5–2–2. Effects of O_3 and salicylic acid (SA) on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II at ambient CO_2 concentration in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure; O^0, $O^{0.1}$, $O^{0.3}$ – 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400} – 400 cm^3 m^{-3} CO_2; S^0, $S^{0.1}$, S^1 – 0, 0.1, 1 mM SA; ●, $O^0 + C^{400} + S^0$; ●, $O^0 + C^{400} + S^{0.1}$; ○, $O^0 + C^{400} + S^1$; ▲, $O^{0.1} + C^{400} + S^0$; ▲, $O^{0.1} + C^{400} + S^{0.1}$; △, $O^{0.1} + C^{400} + S^1$; ■, $O^{0.3} + C^{400} + S^0$; ■, $O^{0.3} + C^{400} + S^{0.1}$; □, $O^{0.3} + C^{400} + S^1$.

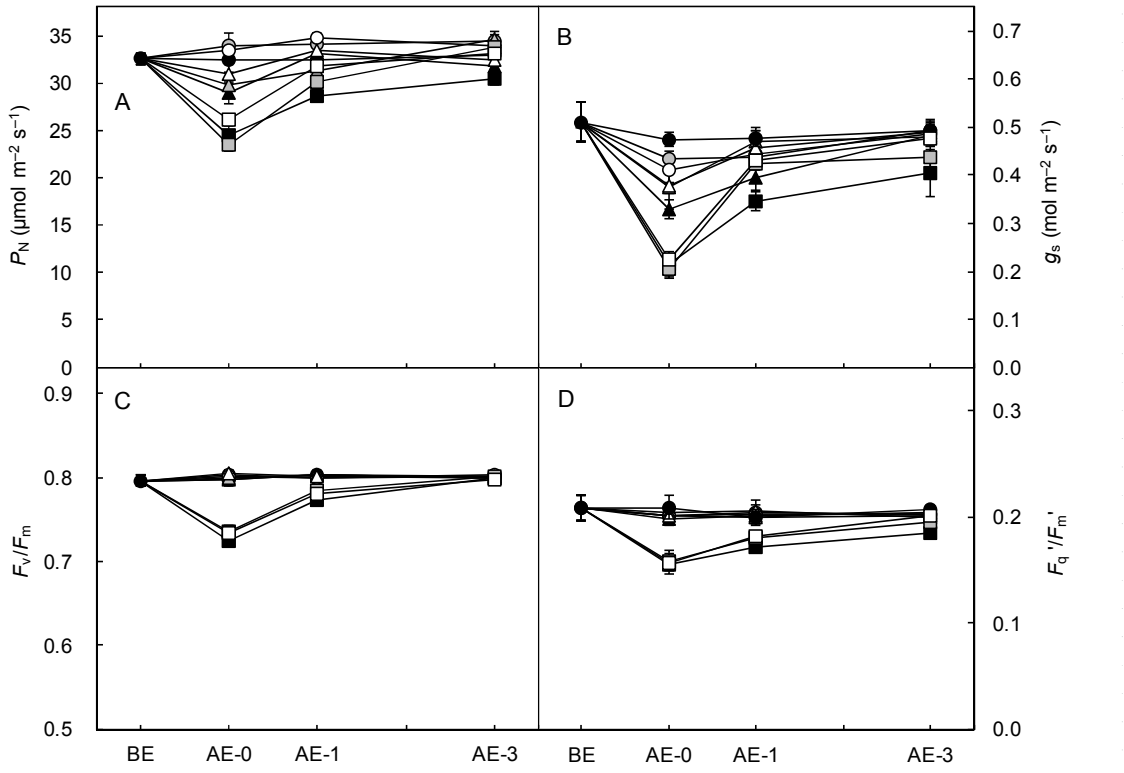

Fig. 5–2–3. Effects of O_3 and SA on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II at elevated CO_2 concentration in rice leaves (Exp. 1). Vertical bars indicate standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure; O^0, $O^{0.1}$, $O^{0.3}$ – 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800} – 400, 800 cm^3 m^{-3} CO_2; S^0, $S^{0.1}$, S^1 – 0, 0.1, 1 mM SA. ●, $O^0 + C^{800} + S^0$; ●, $O^0 + C^{800} + S^{0.1}$; ○, $O^0 + C^{800} + S^1$; ▲, $O^{0.1} + C^{800} + S^0$; ▲, $O^{0.1} + C^{800} + S^{0.1}$; △, $O^{0.1} + C^{800} + S^1$; ■, $O^{0.3} + C^{800} + S^0$; ■, $O^{0.3} + C^{800} + S^{0.1}$; □, $O^{0.3} + C^{800} + S^1$.

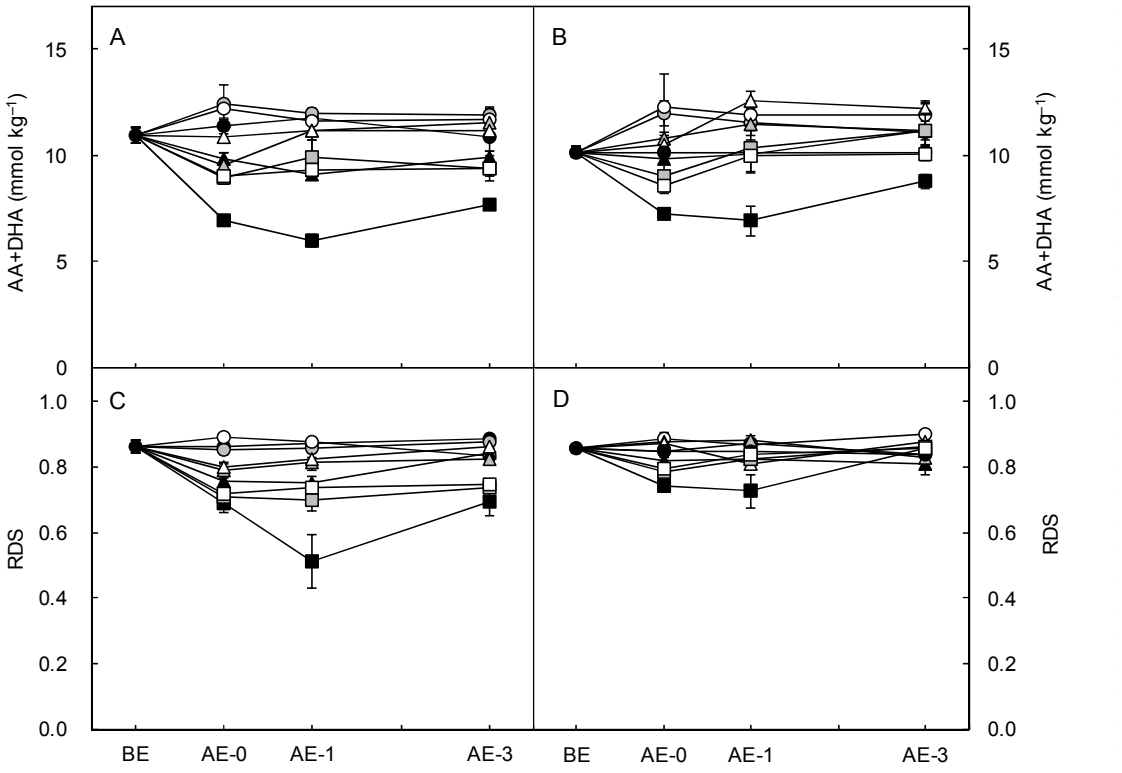

Fig. 5–2–4. Effects of O_3 and SA on total ascorbic acid and redox state of ascorbic acid (RDS) at ambient (A, B) and elevated (C, D) CO_2 concentrations in rice leaves (Exp. 1). Vertical bars indicate standard errors of the means (n = 4). AA, reduced form of ascorbic acid; DHA, dehydroascorbic acid (oxidized form of ascorbic acid); RDS, redox state of ascorbic acid [AA/(AA+DHA)]. BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure; O^0, $O^{0.1}$, $O^{0.3}$ – 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800} – 400, 800 cm^3 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 $C^{800} + S^1$.

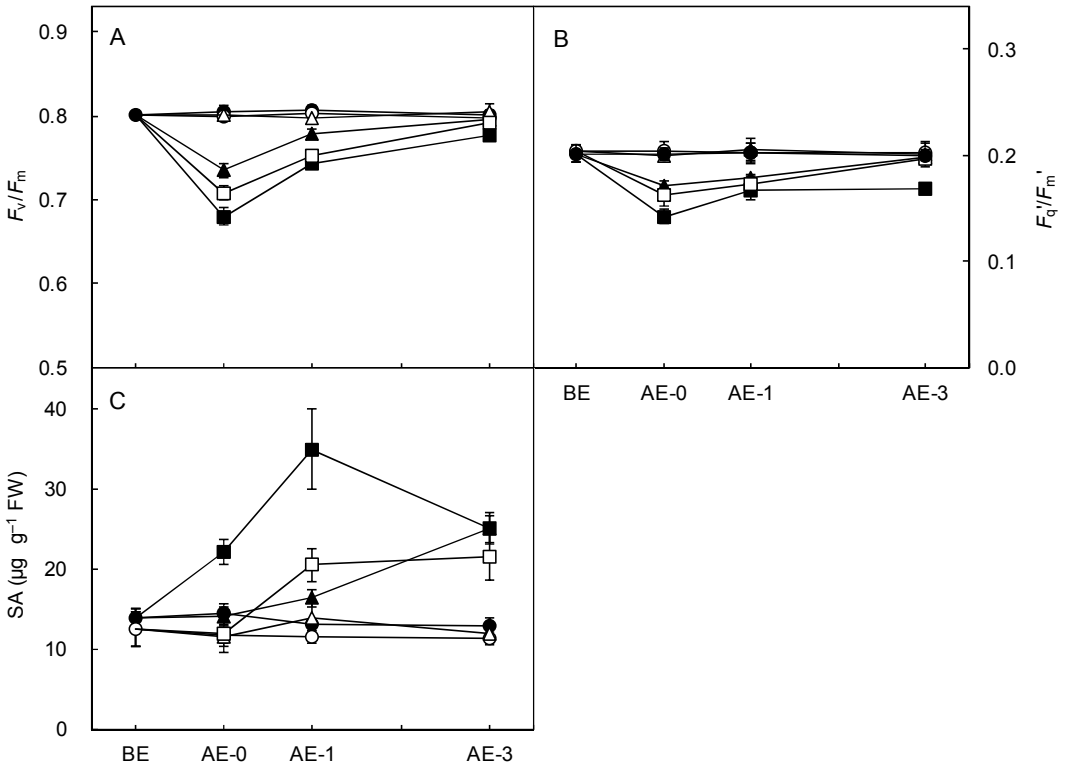

Fig. 5–2–5. Effects of O_3 and CO_2 on maximum (F_v/F_m), operating (F_q'/F_m') quantum efficiency of photosystem II (A, B) and salicylic acid (SA) contents in rice leaves (Exp. 2). Vertical bars indicate standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$ – 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800} – 400, 800 cm^3 m^{-3} CO_2; ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{800}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

Table 5–2–1. 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. 5–2–2, 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.

Table 5–2–2. Statistical analysis of the effects of O_3 and CO_2 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. 5–2–5 (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	***	*	**
	$O_3 \times CO_2$	**	n.s.	n.s.
AE-1	O_3	***	**	***
	CO_2	n.s.	n.s.	*
	$O_3 \times CO_2$	n.s.	n.s.	n.s.
AE-3	O_3	n.s.	n.s.	***
	CO_2	n.s.	n.s.	**
	$O_3 \times CO_2$	n.s.	n.s.	*

Section 3

Relation between O_3-Inhibition of Photosynthesis and Ethylene in Paddy Rice Grown with Different CO_2 Concentrations

5 – 3– 1. Introduction

Ethylene is the simplest olefin synthesized from methionine through intermediates S-adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). In higher plants, ethylene production increases during leaf abscission, flower senescence and fruit ripening. In addition, ethylene biosynthesis increases under stress conditions such as drought, flooding, chilling and mechanical wounding (Taiz and Zeiger, 2010). The O_3-exposure increases ethylene production in leaves of *Arabidopsis* (Rao et al., 2002), pea (Mehlhorn et al., 1991) and tobacco (Mehlhorn et al., 1991; Ogawa et al., 2005b).

To clarify the role of ethylene to O_3-inhibition in rice leaves, the author evaluated effects of exogenous ethylene-generating agent (ethephon) and ethylene action inhibitor (STS) on O_3-inhibition of photosynthesis and ascorbic acid, and quantified ethylene production from leaves under different CO_2 concentrations.

5 – 3– 2. Materials and methods

Plant materials, gas exposure treatments, and ethylene-related plant growth-regulator applications

On April (Exp. 1) and July (Exp. 2), japonica rice seeds (*Oryza sativa* L. cv. Koshihikari) were sown directly in Wagner pots (1/5000 a) and plants were grown in natural-light gas-exposure chamber (S-2003A; Koito Industries, Ltd., Yokohama, Japan) as shown in Chapter 3 Section 4. In Exp. 1, immediately after full expansion of the 8th leaves (Haun index = 8.0; Haun, 1973) solutions of 0.1 % ethephon (Nissan Ethrel 10; Nissan Chemical Industries Ltd., Tokyo, Japan) or 2 mM STS (K-20C; Chrysal Japan Ltd., Oosaka, Japan) was applied to the plants. Ethephon was

sprayed to the shoots using a mister, and STS was applied to the soil and subjected to absorb from the roots. One day after ethephon or STS application (Exp. 1), or immediately after full expansion of the 8th leaves (Exp. 2), rice plants were exposed to O_3 (O^0, $O^{0.1}$, and $O^{0.3}$) during 5-hr local daytime (08:00–13:00) under growth CO_2 concentrations. O_3 and CO_2 were supplied, injected and measured as shown in Chapter 2.

Gas-exchange measurements

In Exp. 1, using portable gas-exchange measurement system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA), gas-exchange in the middle portion of 8th leaves was measured *in situ* at BE, AE-0, AE-1 and AE-3 for four replicate plants in each treatment as shown in Chapter 3 Section 1 under growth CO_2 concentrations.

Chlorophyll fluorescence measurements

In both Exp. 1 and Exp. 2, using a portable fluorometer (MINI-PAM; Heinz Walz GmbH Effeltrich, Germany), the chlorophyll fluorescence of PSII in the 8th leaves was measured simultaneously with the gas exchange measurements for four replicate plants in each treatment as shown in Chapter 3 Section 2.

Ascorbic acid measurements

In Exp. 1, four 8th 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 FW and leaf area, the leaves were frozen in liquid N_2 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) as shown in Chapter 3 Section 1.

Ethylene production measurements

In Exp. 2, four 8th leaves for each treatment were sampled and used for measurements of ethylene production simultaneously with chlorophyll fluorescence measurements. Immediately after measurements of the FW, leaves were sealed in test tube including 4 mL water. They were incubated at 28 °C for 4–hr using an incubator (LTI-700; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Thereafter, the gas sample in test tube was injected into gas chromatography (GC-2010; Shimadzu Corp., Kyoto, Japan) with flame ionization detector (FID-2010Plus; Shimadzu Corp., Kyoto, Japan) using gas-tight syringe (MS-GANX00; Ito Corp., Shizuoka, Japan). The separation of ethylene was conducted with a capillary column (30 m × 0.25 mm × 0.25 μm: Rxi®-1 ms; Restek Corp., Pennsyvania, U.S.) and He as a carrier gas. Temperature of injection port and detector were maintained at 250°C and 280°C, respectively. The column temperature was kept at 170°C for 5 min. The retention time of ethylene was 1.392 min.

Statistical analysis

Statistical analyses were conducted as shown in Chapter 5 Section 1.

5 – 3– 3. Results

Effect of ethylene on O_3-induced visible symptoms in adaxial leaf surface (Exp. 1)

At AE-3, O_3-induced visible symptoms have appeared in the $O^{0.1}$ + C^{400}, $O^{0.3}$ + C^{400}, and $O^{0.3}$ + C^{800} plants. Elevated CO_2 (C^{800}) and STS application ameliorated but ethephon application slightly worsened the leaf symptoms (data not shown).

Effect of ethylene on O_3-inhibition of photosynthesis under ambient CO_2 (Exp. 1)

Photosynthesis-related parameters in the O^0 plants were unaffected by ethephon or STS application during AE-0 to AE-3 under both C^{400} and C^{800} (Figs. 5–3–1 and 2). At AE-0, P_N in the $O^{0.1}$ + C^{400} and $O^{0.3}$ + C^{400} plants were 52% and 24%, respectively, of those at BE. Thereafter, the

decreases persisted: At AE-3, the former and the latter plants were 47% and 21% of those at BE. The O_3-inhibition of P_N in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants slightly ameliorated by STS: At AE-0, P_N in the $O^{0.3} + C^{400}$ + S plants was significantly higher than that in in the $O^{0.3} + C^{400}$ plants and at AE-3, this amelioration was clearer. At AE-3, P_N in the $O^{0.1} + C^{400}$ + S and $O^{0.3} + C^{400}$ + S plants were 81% and 35% of those at BE, respectively. On the other hand, the O_3-inhibition of P_N in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants was not affected or slightly worsened (not significantly different) by ethephon: At AE-3, P_N in the $O^{0.1} + C^{400}$ + E and $O^{0.3} + C^{400}$ + E plants were 43% and 12% of those at BE, respectively (Fig. 5–3–1A). At AE-0, g_s in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants were to 61% and 58% of those at BE, respectively. Thereafter, these plants recovered from O_3-inhibition: At AE-3, g_s in the former and the latter were 72% and 82% of those at BE, respectively. Ethephon and STS did not affect O_3-inhibition of g_s in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants at AE-0 (Fig. 5–3–1B). 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 5–3–1). It also showed interactions between O_3 and ethylene from AE-1 to AE-3 (Table 5–3–1).

At AE-0, F_v/F_m in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants were 78% and 66% of those at BE, respectively. Thereafter, these plants recovered from O_3-inhibition: At AE-3, F_v/F_m in the former and the latter were 93% and 72% of those at BE, respectively. The O_3-inhibition of F_v/F_m in $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants was not affected by ethephon or STS at AE-0, but the $O^{0.3} + C^{400}$ plants were ameliorated by STS at AE-1 and AE-3. The F_v/F_m in the $O^{0.3} + C^{400}$ + S plants at AE-1 and AE-3 were 76% and 88% of those at BE, respectively (Fig. 5–3–1C). At AE-0, F_q'/F_m' in the $O^{0.1} + C^{400}$ and $O^{0.3} + C^{400}$ plants were 73% and 52% of those at BE, respectively. Thereafter, the $O^{0.1} + C^{400}$ plants recovered from O_3-inhibition, but the $O^{0.3} + C^{400}$ plants did not. At AE-3, the $O^{0.1} + C^{400}$ plants recovered fully, but the $O^{0.3} + C^{400}$ plants remained at 55% of BE. The O_3-inhibition of F_q'/F_m' in the $O^{0.3} + C^{400}$ plants was slightly ameliorated by STS. At AE-3, the F_q'/F_m' in the $O^{0.3} + C^{400}$ plants was 75% of that at BE (Fig. 5–3–1D). Three-way ANOVA for F_v/F_m and F_q'/F_m' indicated strong effects of O_3 from AE-0 to AE-3 ($P \leq 0.001$, Table 5–3–1).

Effect of ethylene 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. 5–3–2). At AE-0, P_N in the $O^{0.1} + C^{800}$ and $O^{0.3} + C^{800}$ plants were 85% and 43% of those at BE, respectively. At AE-3, P_N recovered fully in the $O^{0.1} + C^{800}$ plants, but $O^{0.3} + C^{800}$ plants remained at 68% of BE. The O_3-inhibition of P_N in the $O^{0.3} + C^{800}$ plants worsened by ethephon but ameliorated by STS: At AE-3, P_N in the $O^{0.3} + C^{800}$ + E and $O^{0.3} + C^{800}$ + S plants were 44% and 81% of those at BE, respectively (Fig. 5–3–2A). At AE-0, g_s in the $O^{0.1} + C^{800}$ and $O^{0.3} + C^{800}$ plants were 47% and 39% of those at BE, respectively. The O_3-inhibition of g_s in the $O^{0.3} + C^{800}$ plants slightly worsened by ethephon but ameliorated by STS. The g_s in the $O^{0.3} + C^{800}$ + S plants at AE-0 was 60% of that at BE, and the $O^{0.3} + C^{800}$ + E plants at AE-3 was 70% of that at BE (Fig. 5–3–2B). Three-way ANOVA for P_N and g_s showed clear interactions between O_3 and CO_2 during AE-0 to AE-3 ($P \leq 0.001$ or 0.05, Table 5–3–1).

The F_v/F_m in the $O^{0.1} + C^{800}$ and $O^{0.3} + C^{800}$ plants at AE-0 were 93% and 84% of those at BE, respectively. At AE-1, the $O^{0.1} + C^{800}$ plants fully recovered from O_3-inhibition, but the $O^{0.3} + C^{800}$ plants at AE-1 and AE-3 remained 83% and 95% of BE, respectively. Ethephon worsened the O_3-inhibition of F_v/F_m in the $O^{0.1} + C^{800}$ plants at AE-0. On the other hand, the O_3-inhibition of F_v/F_m in the $O^{0.3} + C^{800}$ plants at AE-3 was ameliorated by STS. At AE-3, F_v/F_m in the $O^{0.3} + C^{800}$ + S plants fully recovered from O_3-inhibition (Fig. 5–3–2C). The F_q'/F_m' in the $O^{0.3} + C^{800}$ plants at AE-0 decreased to 81% of that at BE. At AE-1, they recovered fully from O_3-inhibition. The O_3-inhibition of F_q'/F_m' in the $O^{0.1} + C^{800}$ and $O^{0.3} + C^{800}$ plants were not affected by ethaphon or STS (Fig. 5–3–2D). Three-way ANOVA for F_v/F_m and F_q'/F_m' indicated clear interactions between O_3 and CO_2 from AE-0 to AE-3 ($P \leq 0.001$ or 0.05, Table 5–3–1).

Effects of O_3 and ethylene on ascorbic acid contents under different CO_2 concentrations (Exp. 1)

Because the effects of O_3 and ethylene on total ascorbic acid (AA + DHA), AA, and DHA contents expressed per FW were similar to those expressed per leaf area, Fig. 5–3–3 shows values obtained on a FW basis.

At AE-0, total ascorbic acid content in the $O^{0.3} + C^{800}$ plants decreased to 85% of that at BE. At AE-1, the content decreased further. Total ascorbic acid contents in the $O^{0.1} + C^{400}$, $O^{0.1} + C^{800}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants were, respectively, 58%, 55%, 93%, and 69% of those at BE. At AE-3, the content in the $O^{0.1} + C^{800}$ plants recovered fully but in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants, the recoveries remained to low levels (–16%, 31%, and 4% of those at BE). The O_3-inhibition of total ascorbic acid content was not affected by ethephon but ameliorated by STS. Ascorbic acid content in the $O^0 + C^{400}$ plants increased to 133–161% by STS from AE-0 to AE-3 (Fig. 5–3–3A, B).

At AE-0, the redox state of ascorbic acid (RDS) in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants decreased to 92%, 88%, and 91% of those at BE. At AE-1, RDS decreased further: RDS in the $O^{0.1} + C^{400}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants were 75%, 62%, and 85% of those at BE, respectively. Thereafter, the plants recovered from O_3-inhibition at AE-3. The O_3-inhibition of RDS was ameliorated by STS at AE-1 under ambient CO_2 (C^{400}). At AE-1, the $O^{0.1} + C^{400} + S$ plants recovered fully, but the $O^{0.3} + C^{400} + S$ plants remained at 79% of BE (Fig. 5–3–3C, D). Three-way ANOVA for total ascorbic acid contents and RDS showed clear negative effects of O_3 during AE-0 to AE-3 ($P \leq 0.001$, Table 5–3–1). In addition, because STS ameliorated the negative effect of O_3 on ascorbic acid contents and RDS, three-way ANOVA for those showed clear effects of ethylene during AE-0 to AE-3, except RDS at AE-3 (Table 5–3–1).

Effects of O_3 and CO_2 on ethylene production and photosystem II (Exp. 2)

The trends of F_v/F_m and F_q'/F_m' resemble to 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 decreased to 85%, 63%, and 67% of those at BE. The F_q'/F_m' in the $O^{0.1} + C^{400}$, $O^{0.1} + C^{800}$, $O^{0.3} + C^{400}$, and $O^{0.3} + C^{800}$ plants decreased to 66%, 75%, 55%, and 56%

of those at BE. Thereafter, the F_v/F_m and F_q'/F_m' recovered, more or less, from O_3-inhibition: At AE-3, $O^{0.1}$ + C^{400} and $O^{0.3}$ + C^{800} plants recovered fully, but the $O^{0.3}$ + C^{400} plants remained inhibition (86% and 79% of those at BE, respectively) (Fig. 5–3–4A, B).

At AE-0, the ethylene production increased by O_3: The production in the $O^{0.3}$ + C^{400} and $O^{0.3}$ + C^{800} plants at AE-0 increased drastically (302% and 389%, respectively) of those at BE, and in the $O^{0.1}$ + C^{400} and $O^{0.1}$ + C^{800} plants, increased slightly ($P \leq 0.10$). Thereafter, the increase disappeared from AE-1 to AE-3 (Fig. 5–3–4C). Two-way ANOVA for ethylene production indicated a clear effect of O_3 at AE-0 ($P \leq 0.001$, Table 5–3–2).

5 – 3– 4. Discussion

The STS application ameliorated the O_3-induced visible injury and O_3-inhibitiona of P_N and PSII (F_v/F_m and F_q'/F_m'), as in the cases of MeJA and SA applications (Chapter 5 Section 1 and 2). In contrast, ethephon application slightly worsened the O_3-inhibition of photosynthesis-related parameters. These results showed that ethylene function as positive regulator of O_3 injury, in contrast to JA (Chapter 5 Section 1) in rice leaves, as seen in *Arabidopsis* (Rao and Davis, 2001). The amelioration of O_3-induced damage of plants by MeJA application is ascribed to the induction of stomatal closure and increased antioxidant capacity (Chapter 5 Section 1). In contrast to MeJA, SA and ethylene-related plant growth regulator (STS and ethephon) application did not affect g_s (Figs. 5–3–1 and 2; Chapter 5 Section 2). Nevertheless, the amelioration of O_3-inhibition of photosynthesis-related parameters by STS was more effective than that by SA, and even equal to that by MeJA. Therefore, ethylene could be an important determinant in O_3-injury.

Ethylene is the simplest olefin synthesized from methionine through intermediates S-adenosyl-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). In higher plants, ethylene production increases during leaf abscission, flower senescence and fruit ripening. In addition, ethylene biosynthesis increases under stress conditions such as drought, flooding, chilling and mechanical wounding (Taiz and Zeiger, 2010). The O_3-exposure increases ethylene

production in leaves of *Arabidopsis* (Rao et al., 2002), pea (Mehlhorn et al., 1991), tobacco (Mehlhorn et al., 1991; Ogawa et al., 2005b) and paddy rice (Ohki et al., 1999). Also, Rao et al. (2002) reported that ethylene-overproducer mutants (*eto1*, *eto3*) were more sensitive to O_3 than wild type. Overmyer et al. (2000) showed that ethylene-insensitive mutant (*ein2*) was more insensitive to O_3 than wild type, and O_3-sensitive mutant (*rcd1*) produced ethylene more than wild type, thereby more sensitive to O_3. Furthermore, the O_3-induced visible leaf symptoms are ameliorated by ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG), application in pinto bean and tobacco (Mehlhorn et al., 1991). In this section, O_3-induced visible leaf symptoms and O_3-inhibition of photosynthesis-related parameters were ameliorated by ethylene action inhibitor, STS, and slightly worsened by ethylene-generating agent, ethephon (Figs. 5–3–1 and 2). With respect to endogenous ethylene, its production in rice leaves increased immediately after O_3 exposure (AE-0), and decreased rapidly at AE-1 (Fig. 5–3–4). These are coincident with the case of *Arabidopsis* (Rao et al., 2002). Ethylene would be important factor for enlargement of O_3 injury in rice leaves.

The inhibition of ethylene action did not affect stomatal function, but slightly increased total ascorbic acid contents and ameliorated O_3-induced decrease of total ascorbic acid and RDS (Fig. 5–3–3). Therefore, the amelioration of O_3-inhibition by STS attributes to the activation of antioxidant capacity, though it does not fully explain. Although, antioxidant ability is important factor for amelioration of O_3 injury (Didyk and Blum, 2011), The results of previous chapter (Chapter 3 Section 3 and Chapter 5 Section 2) showed that the increase of ascorbic acid by exogenous AA and SA could not fully prevent O_3 injury in rice leaves. However, the amelioration of O_3-induced injury by STS application was more effective than by ascorbic acid and SA applications. These results suggest that ethylene affects other factor for preventing O_3 injury. The ethylene induces programmed cell death (PCD) and senescence (Trobacher, 2009). In addition, chronic and acute O_3 exposures induce premature senescence and hypersensitive reaction-like PCD, respectively (Rao and Davis, 2001). Therefore, it predicted that O_3 induced rapid ethylene

production and thereby cell death and/or senescence, later appeared on leaf surface as visible leaf symptoms. Nakamura and Saka (1978) showed that kinetin and benzimidazole applications ameliorated O_3-induced chlorophyll degradation in rice leaves. As these cytokinins suppress leaf senescence in contrast to ethylene (Taiz and Zeiger, 2010), O_3-induced rapid ethylene production may induce premature leaf senescence.

Ethylene production was not affected by elevated CO_2 (Fig. 5–3–4), although Abeles et al. (1992) reported that elevated CO_2 promoted, inhibited, or had no effect depending plant species and tissues. In Chapter 5 Section 2, JA contents in rice leaves slightly decreased by elevated CO_2, as previously reported (DeLucia et al., 2012). These changes of plant hormone contents could alter the physiological responses of plants including the defense response and sensitivity to various stresses. For example, rice plants grown under elevated CO_2 are more susceptible to leaf blast than those grown under ambient CO_2 (Kobayashi et al., 2006). In addition, elevated CO_2-grown soybean plants had higher sensitivity to acute exposures of O_3 because of the lowered antioxidant capacity under elevated CO_2 (Gillespie et al., 2011).

Results of this chapter show that JA and SA function as negative regulators of O_3 inhibition, and ethylene functions as a positive regulator of O_3-inhibition. Also, jasmonates (JA and MeJA) and ethylene increased at AE-0, and SA increased with a lag time (from AE-1 to AE-3) after O_3 exposure. Furthermore, increased amount of jasmonates and ethylene were higher than that of SA. Therefore, it seems that JA and ethylene are more important for the determination of O_3 inhibition than SA in rice leaves. This phenomenon may attribute to high SA contents in rice leaves (8–30 μg g^{-1} FW), which is higher than other plants such as *Arabidopsis* (0.01–0.1 μg g^{-1} FW) (Ogawa et al., 2006). According to Yang et al. (2004), rice had two orders of magnitude higher levels of SA than tobacco and *Arabidopsis,* and appeared to be insensitive to exogenous SA. In addition, Kanno et al. (2012) reported that JA and SA contents in rice leaves increased by feeding damage of white-back planthopper (*Sogatella furcifera*), but the increment of SA was less than that of JA, because rice plant contain much higher levels of endogenous SA in healthy tissues. Therefore, the

author concludes that the role of SA in rice is less than in other plants, and JA and ethylene is major components of signal transduction for determining of O_3 injury.

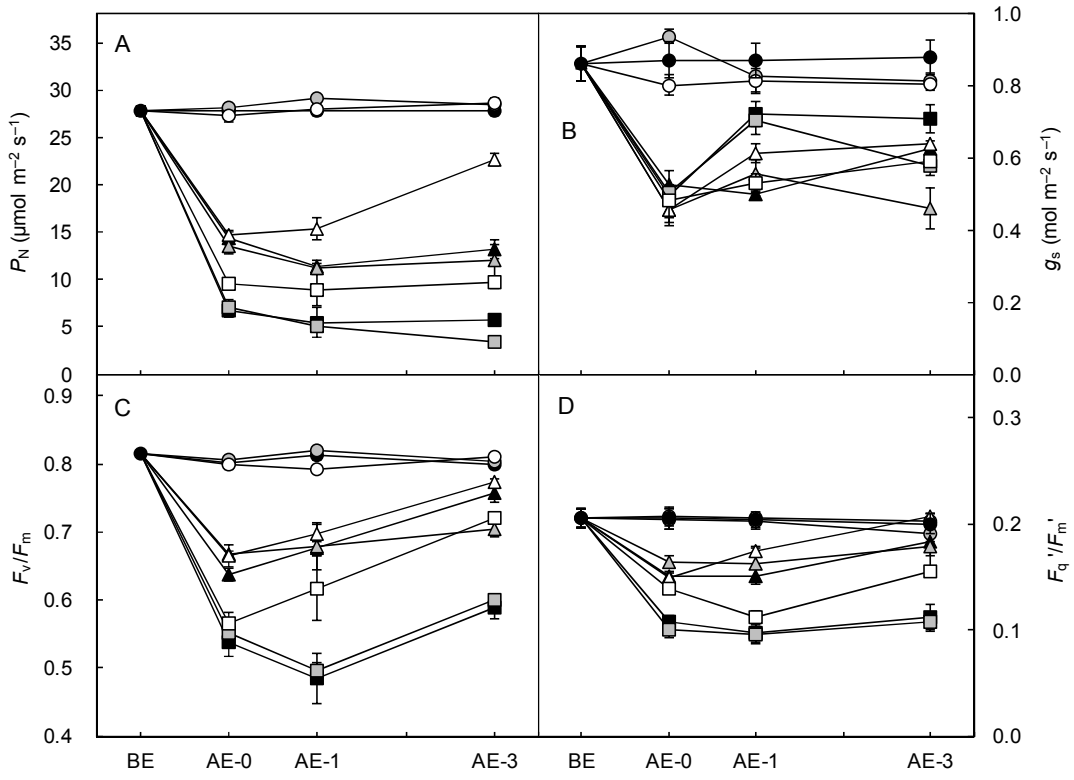

Fig. 5–3–1. Effects of O_3 and ethylene- related plant growth regulator on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII at ambient CO_2 concentration in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}: 400 cm^3 m^{-3} CO_2; E, S: ethephon and STS treatment. ●, $O^0 + C^{400}$; ●, $O^0 + C^{400} + E$; ○, $O^0 + C^{400} + S$; ▲, $O^{0.1} + C^{400}$; ▲, $O^{0.1} + C^{400} + E$; △, $O^{0.1} + C^{400} + S$; ■, $O^{0.3} + C^{400}$; ■, $O^{0.3} + C^{400} + E$; □, $O^{0.3} + C^{400} + S$.

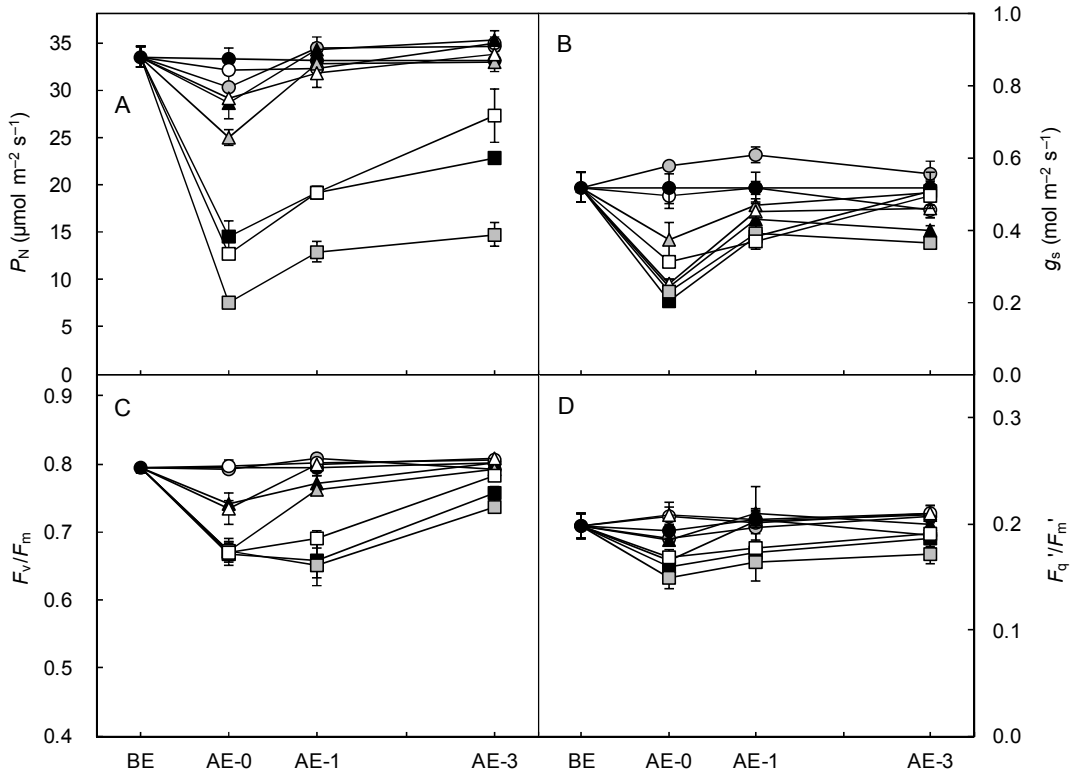

Fig. 5–3–2. Effects of O_3 and ethylene- related plant growth regulator on net photosynthetic rate (P_N), stomatal conductance (g_s), and maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of photosystem II at elevated CO_2 concentration in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{800}: 800 cm^3 m^{-3} CO_2; E, S: ethephon and STS treatment. ●, $O^0 + C^{800}$; ●, $O^0 + C^{800} + E$; ○, $O^0 + C^{800} + S$; ▲, $O^{0.1} + C^{800}$; ▲, $O^{0.1} + C^{800} + E$; △, $O^{0.1} + C^{800} + S$; ■, $O^{0.3} + C^{800}$; ■, $O^{0.3} + C^{800} + E$; □, $O^{0.3} + C^{800} + S$.

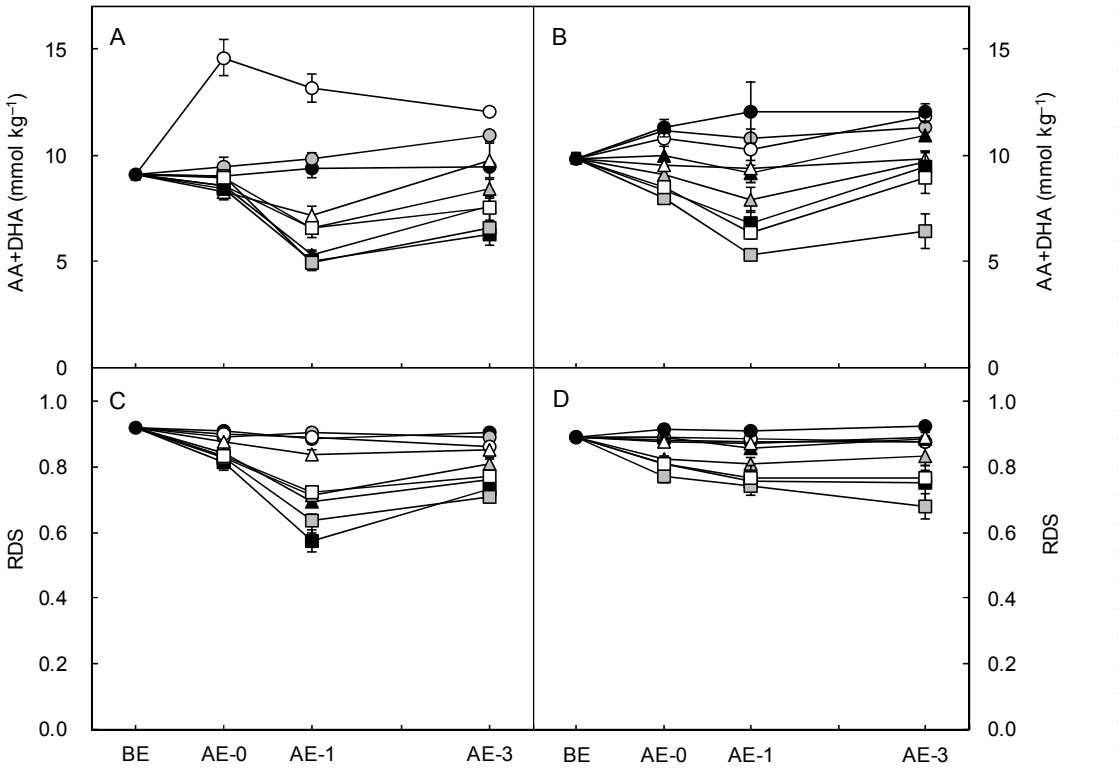

Fig. 5–3–3. Effects of O_3 and ethylene-related plant growth regulator on total ascorbic acid and redox state of ascorbic acid (RDS) at ambient (A, B) and elevated (C, D) CO_2 concentrations in rice leaves (Exp. 1). Vertical bars represent standard errors of the means (n = 4). AA: reduced form of ascorbic acid; DHA: dehydroascorbic acid (oxidized form of ascorbic acid), RDS: redox state of ascorbic acid [AA/(AA+DHA)]. BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{400}, C^{800}: 400, 800 cm^3 m^{-3} CO_2; E, S: ethephon and STS treatment. ●, O^0 + C^{400} or C^{800}; ●, O^0 + C^{400} or C^{800} + E; ○, O^0 + C^{400} or C^{800} + S; ▲, $O^{0.1}$ + C^{400} or C^{800}; ▲, $O^{0.1}$ + C^{400} or C^{800} + E; △, $O^{0.1}$ + C^{400} or C^{800} + S; ■, $O^{0.3}$ + C^{400} or C^{800}; ■, $O^{0.3}$ + C^{400} or C^{800} + E; □, $O^{0.3}$ + C^{400} or C^{800} + S.

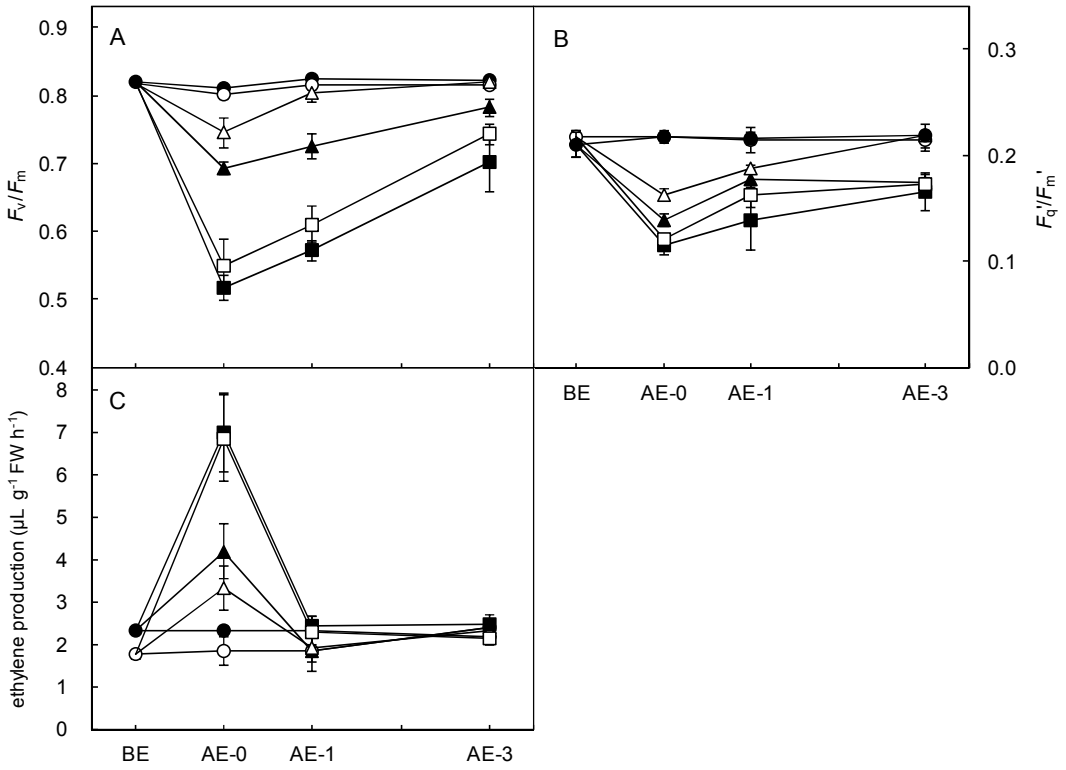

Fig. 5–3–4. Effects of O_3 and CO_2 on maximum (F_v/F_m), operating (F_q'/F_m') quantum efficiency of photosystem II (A, B) and ethylene production in rice leaves (Exp. 2). Vertical bars represent standard errors of the means (n = 4). BE, AE-0, AE-1 and AE-3 respectively denote before, immediately after, 1 d and 3 d after gas exposure. O^0, $O^{0.1}$, $O^{0.3}$: 0, 0.1, 0.3 cm^3 m^{-3} O_3; C^{800}: 800 cm^3 m^{-3} CO_2; ●, $O^0 + C^{400}$; ○, $O^0 + C^{800}$; ▲, $O^{0.1} + C^{400}$; △, $O^{0.1} + C^{400}$; ■, $O^{0.3} + C^{400}$; □, $O^{0.3} + C^{800}$.

Table 5–3–1. Statistical analysis of the effects of O_3, CO_2, and ethylene-related plant growth regulators (STS and ethephon) on net photosynthetic rate (P_N), stomatal conductance (g_s), maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII, and total (AA+DHA), reduced (AA), and oxidized (DHA) ascorbic acid contents per fresh weight in rice leaves shown in Figs. 5–3–1, 2, and 3 (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	RDS
AE-0	O_3	***	***	***	***	***	***
	CO_2	***	***	***	***	n.s.	n.s.
	Ethylene	***	n.s.	n.s.	**	**	*
	O_3×CO_2	***	**	***	***	n.s.	n.s.
	O_3×Ethylene	n.s.	n.s.	n.s.	n.s.	**	n.s.
	CO_2×Ethylene	**	n.s.	n.s.	n.s.	**	n.s.
	O_3×CO_2×Ethylene	n.s.	n.s.	n.s.	*	***	n.s.
AE-1	O_3	***	***	***	***	***	***
	CO_2	***	***	***	***	***	***
	Ethylene	*	n.s.	*	n.s.	**	***
	O_3×CO_2	***	***	***	***	**	***
	O_3×Ethylene	**	*	n.s.	n.s.	n.s.	*
	CO_2×Ethylene	*	n.s.	n.s.	n.s.	***	**
	O_3×CO_2×Ethylene	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AE-3	O_3	***	***	***	***	***	***
	CO_2	***	***	***	***	***	n.s.
	Ethylene	***	*	***	*	**	n.s.
	O_3×CO_2	***	***	***	**	n.s.	n.s.
	O_3×Ethylene	***	**	***	n.s.	n.s.	n.s.
	CO_2×Ethylene	n.s.	*	***	n.s.	***	n.s.
	O_3×CO_2×Ethylene	***	*	***	n.s.	n.s.	n.s.

Table 5–3–2. Statistical analysis of the effects of O_3 and CO_2 on maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiency of PSII, and ethylene production in rice leaves presented in Fig. 5–3–4 (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'	ethylene
AE-0	O_3	***	***	***
	CO_2	n.s.	*	n.s.
	$O_3 \times CO_2$	n.s.	n.s.	n.s.
AE-1	O_3	***	***	n.s.
	CO_2	*	n.s.	n.s.
	$O_3 \times CO_2$	*	n.s.	n.s.
AE-3	O_3	***	***	n.s.
	CO_2	n.s.	n.s.	n.s.
	$O_3 \times CO_2$	n.s.	n.s.	n.s.

Chapter 6

General Discussion

Ozone (O_3) is a health hazard for humans and plants. It attacks all plant species and decreases their photosynthesis and growth rates concurrently with visible symptoms. When exposed to O_3, rice plants (Japan's staple summer crops) suffer damage: inhibition of net photosynthetic rate (P_N) and stomatal conductance (g_s) (Imai and Kobori, 2008), decreased ribulose 1, 5-bisphosphate carboxylase/oxygenase (Ishioh and Imai, 2005), chlorophyll and carotenoid contents (Rai and Agrawal, 2008), in addition to visible leaf-related symptoms (Imai and Kobori, 2008) and breakdown of the cellular ultrastructure (Toyama et al., 1989). Moreover, O_3 suppresses growth (Imai and Ookoshi, 2011), alters photoassimilate partitioning (Nouchi et al., 1995), and decreases grain yield (Reid and Fiscus, 2008; Imai and Ookoshi, 2011).

Concurrent with O_3 air pollution, global atmospheric CO_2 concentration has increased from the pre-industrial value of about 280 $cm^3\ m^{-3}$ to the current level of 391 $cm^3\ m^{-3}$. This trend is expected to continue unless human activities are curtailed substantially (WMO WDCGG 2013). Elevated CO_2 induces various responses such as promotion of photosynthesis, stomatal closure, increase of leaf thickness and yield, and changes of metabolism including plant hormones and antioxidants (Imai, 1988; Gillespie et al., 2011; Kirkham, 2011; DeLucia et al., 2012). Because elevated CO_2 concentrations decrease g_s, they ameliorate O_3-induced injury by suppressing O_3 intake through stomata (Booker and Fiscus, 2005). Therefore, this study was conducted to elucidate the effects of acute (single or repeated) O_3 exposure and elevated CO_2 on rice plant physiology such as photosynthesis, which are the bases for growth and dry-matter production.

6 – 1. Establishment of experimental approach

Because the transient O_3 injury of leaves is lost with time, evaluating O_3 effect on the maximum

quantum efficiency of PSII (F_v/F_m) is difficult. In Chapter 2, the author examined F_v/F_m in rice leaves exposed to different O_3 concentrations (0, 0.1, and 0.3 $cm^3\ m^{-3}$, expressed as O^0, $O^{0.1}$, and $O^{0.3}$) under different dark adaptation periods (0, 1, 5, 10, 20, and 30 min, expressed as D^0, D^1, D^5, D^{10}, D^{20}, and D^{30}) to ascertain its optimum time span. F_v/F_m was inhibited by O_3. However, in the O^0 and $O^{0.1}$ plants, it recovered during dark adaptation. In the $O^{0.3}$ plants, F_v/F_m decreased gradually with time. The F_0 increased by O_3. It increased further in the $O^{0.3}$ plants during dark adaptation. Under high light intensity, F_m was decreased by O_3. The O_3-induced damage to F_v/F_m was therefore more pronounced. However, the F_m sensitivity was lower than that of F_0. Consequently, the damage to PSII was attributed mainly to the inhibition of electron transport from Q_A to Q_B. The F_v/F_m ratio in the O^0 plants was fully recovered at D^{10}. In the $O^{0.1}$ plants, F_v/F_m increased from D^{10} to D^{20}. The effects of O_3 on the xanthophyll cycle-dependent quenching (fast relaxation phase) of q_I disappeared when the dark adaptation period was greater than 20 min. However, it was difficult to distinguish the effects of O_3 and other factors (e.g., light) before D^5. These results demonstrate that the optimum dark adaptation period in rice leaves is 10 min because the effect of O_3 remains maximal, although the effects of other factors on F_v/F_m disappear during this period. By accurate measurement of F_v/F_m, the physiology of O_3 effect on PSII in rice leaves is evaluated precisely.

6 – 2. Effects of O_3 and CO_2 on photosynthesis in leaves of paddy rice

The study of Chapter 3 was conducted to elucidate the interactive effects of O_3 and CO_2 on rice leaves. In Chapter 3 Section 1, gas exchange, chlorophyll fluorescence, ascorbic acid and glutathione were examined under acute (5 h), combined exposures of O_3 (O^0, $O^{0.1}$, and $O^{0.3}$), and CO_2 (400 or 800 $cm^3\ m^{-3}$, expressed as C^{400} or C^{800}, respectively) in natural-light gas-exposure chambers. The P_N, F_v/F_m, and operating quantum efficiency of photosystem II (F_q'/F_m') in young (8^{th}) leaves decreased during O_3 exposure. However, these were ameliorated by C^{800} and fully recovered within 3 d in clean air ($O^0 + C^{400}$) except for the $O^{0.3} + C^{400}$ plants. The maximum PSII

efficiency at 1,500 μmol m^{-2} s^{-1} PPFD (F_v'/F_m') for the $O^{0.3}$ + C^{400} plants decreased for all measurement times, probably because leaves with severely inhibited P_N also had severely damaged PSII. The P_N of the flag (16^{th}) leaves at heading decreased under O_3 exposure, but the decline was smaller and the recovery was faster than that of the 8^{th} leaves. The F_q'/F_m' of the flag leaves in the $O^{0.3}$ + C^{400} and $O^{0.3}$ + C^{800} plants decreased immediately after gas exposure, but the F_v/F_m was unaffected. These effects indicate that elevated CO_2 interactively ameliorated the inhibition of photosynthesis induced by O_3 exposure. However, changes in antioxidant levels did not explain the interaction above. Furthemore, antioxidant (ascorbic acid and glutathione) levels in flag leaves were higher than those in 8^{th} leaves.

The results presented in Chapter 3 Section 1 indicate that the difference of O_3-inhibition of photosynthesis between the 8^{th} leaves and flag leaves are ascribable to their respective leaf thickness and leaf inclination angle. Therefore, as explained in Chapter 3 Sections 2 and 3, the author evaluated the respecitive effects of the leaf inclination angle and antioxidant level on the leaf injury of rice by O_3. To evaluate the effect of inclination angle of the leaf on the leaf injury of rice by O_3, the effect of a 5-h exposure to O_3 (O^0, $O^{0.1}$ or $O^{0.3}$) of the flag leaf was examined in Section 2. The middle portion of the flag leaf was set horizontally using lead weights (H plant) and was compared with a non-treated, erect leaves (E plant). Results show that P_N, g_s and F_q'/F_m' were decreased by exposure to O_3. Furthermore, the inhibitory effects of $O^{0.1}$ and $O^{0.3}$ on the H plants were severer than on the E plants. The F_v/F_m in the leaf of H plants decreased immediately after exposure to O_3. These results demonstrate that the weaker inhibitory effect of O_3 on the erect leaf depends on the lower light intensity at the leaf surface, rather than the horizontal leaf position.

In Section 3, the effect of exogenous ascorbic acid on O_3-inhibition of photosynthesis is examined. Before a 5-h exposure to O_3 (O^0, $O^{0.1}$ or $O^{0.3}$), ascorbic acid was applied to the plants. The O_3-inhibition of P_N, F_v/F_m and F_q'/F_m' in $O^{0.1}$ plants was ameliorated by ascorbic acid application. However, those in $O^{0.3}$ plants were unaffected by ascorbic acid application. In addition, the total ascorbic acid content was decreased by O_3, and increased by ascorbic acid

application. Therefore, the negative effect of O_3 on total ascorbic acid was compensated by ascorbic acid application. These results (Chapter 3 Sections 1 and 3) showed that the endogenous ascorbic acid was the determinant for O_3-inhibition of photosynthesis in rice leaves. The differences of O_3-inhibition of photosynthesis between leaf positions were ascribable to their respective leaf thickness and leaf inclination angle (Chapter 3 Sections 1–3).

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 O_3 exposure to rice that is grown at elevated CO_2. In Section 4, the author examined the effect of acute exposure to O_3 (O^0, $O^{0.1}$ or $O^{0.3}$) on leaf photosynthesis of paddy rice grown under different CO_2 concentration (C^{400} and C^{800}). The P_N, F_v/F_m, and F_q'/F_m' decreased immediately after O_3 exposure, but these were ameliorated by C^{800}, as in the case of short CO_2 treatment. The acclimation of g_s to elevated CO_2 occurred in rice leaves of C^{800} plants. Therefore, it was anticipated that O_3-induced injury of photosynthesis in the $C^{800} + O^{0.3}$ plants was more suppressed than in $C^{400} \rightarrow C^{800} + O^{0.3}$ plants (grown at $C^{400} + O^0$ and received acute $C^{800} + O^{0.3}$ 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 (DHA), and redox state of ascorbic acid (RDS) were unaffected by C^{800} when they were expressed as a unit per leaf area. However, the total ascorbic acid and AA contents per fresh and dry mass bases were decreased by C^{800} because the specific leaf weight (SLW) was increased considerably by C^{800}. Therefore, long-term exposure of rice plants to elevated CO_2 might have little effect on antioxidant systems.

6 – 3. Effects of O_3 and CO_2 on nitrogen metabolism in leaves of paddy rice

Nitrogen metabolism is particularly important for plant growth because it regulates photosynthesis and the C/N ratio. Once the nitrogen metabolism is inhibited, the production of photosynthesis-related nitrogen compounds, i.e., chlorophyll and Rubisco, decrease. In contrast,

once the photosystem is inhibited, the reducing power is limited and the NiR receives negative effects. The study described in Chapter 4 demonstrated the interaction between O_3 and CO_2 on photosynthesis and nitrogen metabolism in rice. Measurements were conducted for photosynthesis (F_v/F_m and F_q'/F_m'). A key enzyme related to nitrogen metabolism in leaves, nitrate reductase (NR), catalyzes the conversion from NO_3^- to NO_2^- using NADH and nitrite reductase (NiR), from NO_2^- to NH_4^+ using Fd.

Results show that O_3 inhibited F_v/F_m, F_q'/F_m', and NiR activity, but they were ameliorated by elevated CO_2, suggesting that the O_3-inhibition of photosynthesis accompanied the reduced electron transport toward the downstream of the photosystem and suggesting that the reduction power became deficient. Because NiR locates in the chloroplast and uses reducing power from PSII, it must receive a greater O_3-inhibition than cytoplasmic NR does. In addition, SiR in sulfur metabolism and chloroplast GOGAT might be influenced by O_3 because they use Fd as a reducing power, as in the case of NiR (Buchanan et al., 2000; Taiz and Zeiger, 2010). Furthermore, because elevated CO_2 singly affect the C/N ratio of plants (Imai, 1988), plants in the future are expected to be affected by the complex effects of O_3 and CO_2.

6 – 4. Relations between O_3-inhibition of photosynthesis and plant hormones

Chapter 5 demonstrated the effects of plant hormones, namely jasmonic acid (JA), salicylic acid (SA), and ethylene on the O_3-inhibition of photosynthesis in rice leaves. Section 1, 2, and 3 examined the relation between O_3-inhibition of photosynthesis and JA, SA and ethylene, respectively, in paddy rice grown with different CO_2 concentrations. All sections (Chapter 5 Section 1, 2, and 3) examined the effects of exogenous plant hormones and quantified endogenous plant hormone contents in rice leaves.

The role of JA on O_3 (O^0, $O^{0.1}$ and $O^{0.3}$)-induced photosynthetic inhibition of rice leaves at different CO_2 concentrations (C^{400} and C^{800}) was evaluated in Section 1. Methyl jasmonate (MeJA) solutions (0, 10, 100 μM; abbreviated respectively as M^0, M^{10}, and M^{100}) were applied

one day before acute exposure (5 h) of O_3. MeJA ameliorated the O_3-inhibition of F_v/F_m and F_q'/F_m' of PSII. Rapid recovery from O_3-inhibition of P_N was observed after the MeJA application, but MeJA decreased the P_N and g_s at AE-0. The O_3 decreased the total ascorbic acid content, although the MeJA increased it. The effects of MeJA on the O_3-inhibition of PSII and ascorbic acid content at elevated CO_2 concentration (C^{800}) were less than at ambient concentration (C^{400}). With respect to endogenous JA and MeJA, MeJA contents in rice leaves were increased immediately after O_3 exposure, and decreased from AE-1 to AE-3, although JA contents were increased with a lag time. Therefore, it was predicted that O_3 induced JA biosynthesis. Thereafter JA was converted MeJA.

The role of SA on acute O_3 (O^0, $O^{0.1}$ and $O^{0.3}$)-induced photosynthetic inhibition of paddy rice leaves given different CO_2 concentrations (C^{400} and C^{800}) was evaluated in Section 2. Salicylic acid solutions (0, 0.1, and 1 mM; S^0, $S^{0.1}$, and S^1, respectively) were applied as a pretreatment one day before O_3 exposure. Salicylic acid ameliorated the O_3-inhibition of P_N slightly, and O_3-induced depletion of total ascorbic acid and RDS substantially. Salicylic acid did not increase P_N in non-treated leaves (O^0 plants). However, O_3 exposure elevated the level of endogenous SA. These results show that SA plays a vital role in the defense response to acute O_3 exposure in paddy rice. Effects of SA on O_3-inhibition of PSII and ascorbic acid content were unaffected by elevated CO_2 (C^{800}).

The role of ethylene on acute O_3 (O^0, $O^{0.1}$ and $O^{0.3}$)-induced photosynthetic inhibition of paddy rice leaves, given different CO_2 concentrations (C^{400} and C^{800}), is presented for evaluation in Section 3. Ethephon and STS were applied as a pretreatment one day before O_3 exposure. The STS application ameliorated the O_3-induced visible leaf symptom and O_3-inhibition of photosynthesis, but ethephon application worsened it slightly or did not affect it. In addition, the effects of O_3 and CO_2 on ethylene production from rice leaves were evaluated. The O_3 exposure elevated the ethylene production from leaves at AE-0. The increase in ethylene production disappeared from AE-1. These results demonstrate that ethylene is an important signal

transduction component for the enlargement of O_3 injury in paddy rice.

Chapter 5 Sections 1–3 demonstrate that JA and SA function as negative regulators of O_3 inhibition, and that ethylene functions as a positive regulator as in the case of *Arabidopsis*. However, the role of SA in rice leaves apparently differs from that in *Arabidopsis*. In *Arabidopsis*, SA increased immediately after O_3 exposure (Rao et al., 2000). In contrast, the present study showed that SA content was greater by O_3 at AE-1 and AE-3 than at AE-0. Therefore, SA might function in downstream of JA and ethylene. Furthermore, the role of SA might have a minor function in rice leaves compared to other plants. In summary, JA and ethylene are major determinant for O_3 injury in rice leaves.

6 – 5. Models of O_3 inhibition in leaves of paddy rice

The author inferred the mechanism of O_3-inhibition in rice leaves using results presented in all chapters. The first schematic model assessed O_3-induced damage of photosynthesis (Fig. 6–1). The O_3 induces the decrease of P_N through (1) oxidative damage and (2) stomatal closure. The former (oxidative damage) includes the decrease of Rubisco activity and inactivation of the photosystem. The inactivation of the photosystem, especially PSII, induces a lack of NADPH and ATP used for carbon fixation reaction, and thereby degrades the carbon fixation reaction. The deterioration of P_N through the lack of an energy supply induces increased C_i, and thereby induces stomatal closure. The O_3-induced stomatal closure is attributed to two factors, i.e., (1) indirect factor through the increased C_i and (2) direct factor. The latter factor means that O_3 damages the stomatal function (Torsethaugen et al., 1999). In this study, g_s in the darkness was abnormally high when plants were exposed to high concentration of O_3, implying the existence of a direct factor. The stomatal closure decreases O_3 uptake through stomata. However, stomatal closure is accompanied by decrease C_i, indicating the absence of a substrate for carbon dioxide fixation. Similarly, the inhibition of P_N induces excessive energy if the light intensity is sufficiently high for plants. Excessive energy increases ROS production, and thereby induces oxidative stress. The

greater the light intensity, the greater the excessive energy becomes. Therefore, the leaf inclination angle determines the intercepted radiation. Moreover, it is a determinant for the O_3-inhibition of photosynthesis. In short, the balance of energy and stomatal aperture are important determinants for O_3-inhibition of P_N.

Elevated CO_2 induces increased C_i and stomatal closure. The increased C_i promotes P_N and thereby decreases excess energy. Although stomatal closure induces a decrease in P_N under ambient CO_2 level, the elevated CO_2 increases C_i at a higher level. Thereby P_N is maintained or even promoted. Simultaneously, stomatal closure under elevated CO_2 decreases the O_3 intake through stomata, thereby suppressing the oxidative damage. Therefore, elevated CO_2 ameliorates O_3-inhibition.

The second schematic model assesses the relation between plant hormone and O_3 injury (Fig. 6–2). Ozone rapidly induces jasmonic acid (JA) and ethylene biosynthesis in rice leaves. The JA functions as a positive regulator for the induction of O_3 injury, inducing the activation of antioxidant biosynthesis and stomatal closure. The induction of antioxidant metabolism decreases the ROS level and, consequently, the oxidative damage. Similarly, stomatal closure by JA suppresses the O_3 intake through stomata. Therefore, JA-induced activation of antioxidant biosynthesis and stomatal closure suppresses O_3 injury. However, JA decreases P_N through stomatal closure. Because JA induces the decrease in P_N and chlorophyll content, JA seems to act as a positive regulator for leaf senescence. These results demonstrate that JA functions not only for the induction of defense responses but also for the regulation of balance of photosynthesis and growth. Ethylene functions as a negative regulator for the induction of O_3 injury. Because ethylene action inhibitor (STS) application suppresses O_3-induced visible leaf symptoms, ethylene seems to induce cell death and/or senescence. Similarly, ethylene might induce ROS synthesis and suppress antioxidant metabolism. Both JA and ethylene increase immediately after O_3 exposure, but SA increases far later than the former two. Therefore, these results do not predict whether the direct factor (O_3) or indirect factor (JA and/or ethylene) induces SA biosynthesis. Although SA promotes

antioxidant metabolism in O_3-exposed rice leaves, its role is lighter than in other plants such as *Arabidopsis*. In short, JA and ethylene are important determinants for O_3 injury in rice leaves. Additionally, it appears that the predicted mechanisms for O_3 injury in model plants (Rao and Davis, 2001; Kangasjarvi et al., 2005) do not necessarily agree with those of other crops.

6– 6. Concluding remarks and future prospects

By conducting this series of studies, the author recognizaed that rice response to O_3 is extremely complex as in model plants (e.g. *Arabidopsis*), although the different feature between the two were found (e.g. the role of SA). Furthermore, rice responses differ among leaf positions and growth stages, and change depending on environmental conditions (e.g., CO_2 concentration and light intensity). Therefore, to maintain rice yields under a high concentration O_3 concentration, it is necessary to tackle these problems, which depend on the growth stage, leaf age, and season.

Based on the results of this study, the author proposes (1) the genetic approcach and (2) a cultural approach as countermeasure against O_3 stress. The former (1) is to develop mutans producing defense components (e.g., JA, SA, and antioxidants). Such mutants would suppress O_3 damage as in *Arabidopsis*. However, the overproduction of defence components in normal conditions (non-stressed conditions) might suppress growth rate and yield, and alter other processes (e.g. flowering): Jasmonates (i.e., JA and MeJA) induce defense responses to various stresses but promote senescence, and thereby worsen the production and yield. Furthermore, the generation of cultivar that has low intercepted radiation might suppress O_3 injury, but decreased intercepted radiation degrades photosynthetic capability. The latter (2) is to apply defense components to rice plants. However, if the timing of application is inappropriate, then it might suppress photosynthesis and growth in non-stressed plants. Plant hormones such as JA and SA induce not only defense responses but also other responses. Therefore, it seems that direct defense components such as antioixidants are beneficial for agriculture. From the viewpoint of economic perspective, antioxidants are of more benefit than plant hormons. The O_3 damage differs between

acute and chronoic exposures. Actually, rice plants might receive acute and chronic O_3 exposures in paddy field. Therefore, as a basis of stable supply of paady rice in Japan, it is necessary to understand rice responses to the chronic O_3 exposure and the combination of aucte and chronic O_3 exposures, in addition to acute O_3 exposure. Furthermore, it is necessary to predict exposure patterns of O_3 in paddy rice fields in the future.

Finally, it is necessary to establish practical countermeasures to O_3 stress of paddy rice as well as other crop species based on results of existing studies including this study and further studies.

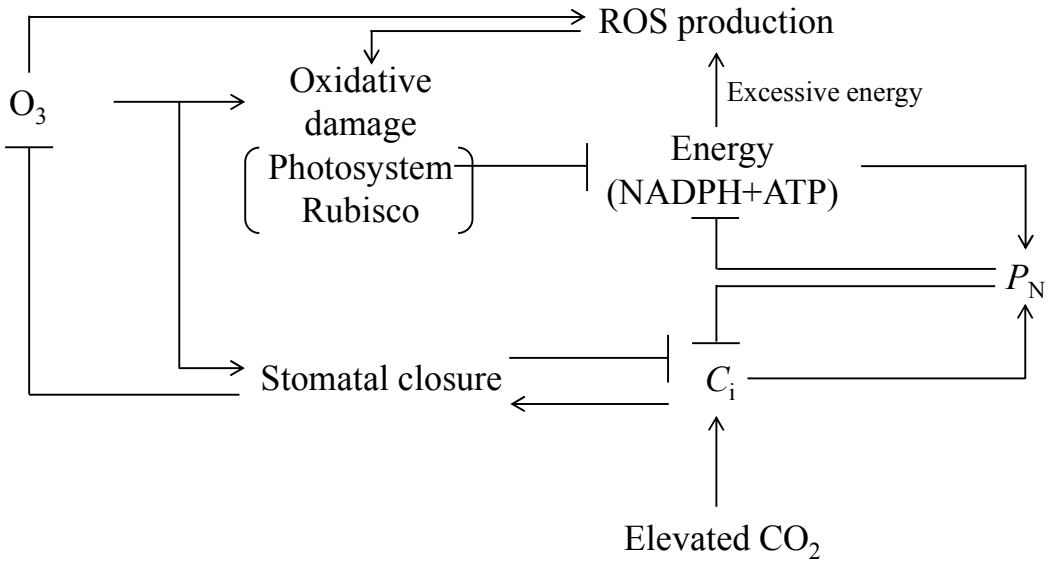

Fig. 6–1. A model of O_3-induced damage of photosynthesis in rice leaves. P_N, net photosynthetic rate; C_i, intercellular CO_2 concentration; ROS, reactive oxygen species; ↑, promotion or induction; T, inhibition or suppression.

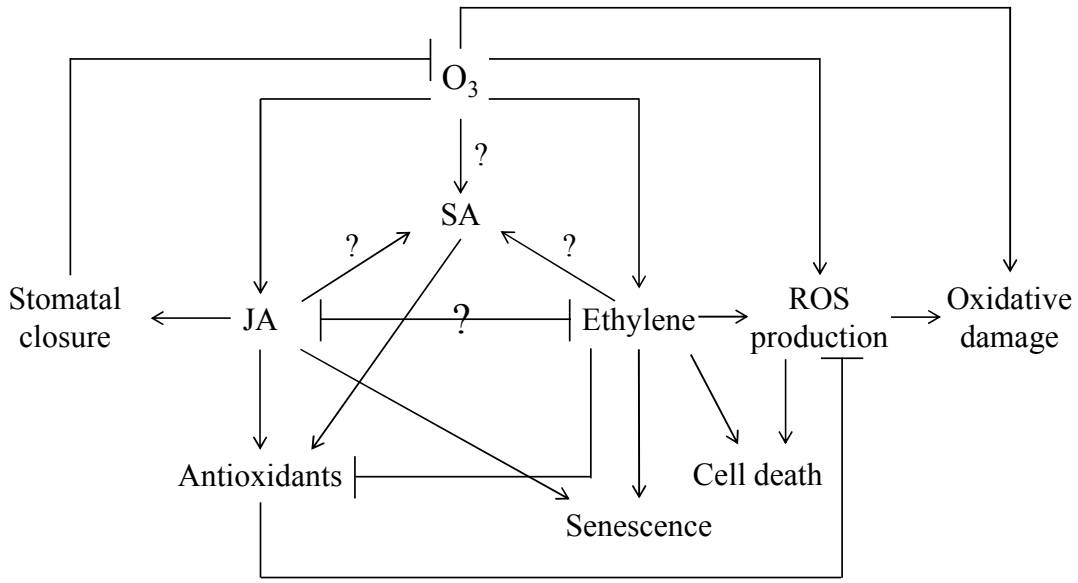

Fig. 6–2. A model of plant hormone action mechanisms in O_3-exposed rice leaves. O_3, ozone; JA, jasmonic acid; SA, salicylic acid; ROS, reactive oxygen species; ↑, promotion or induction; ⊤, inhibition or suppression.

Summary

Ozone (O_3) attacks all terrestrial plants. It decreases their photosynthesis and thereafter induces visible injury that accompanies the growth inhibition and yield reduction. Concurrently with O_3 air pollution, the global atmospheric carbon dioxide (CO_2) concentration has increased from the pre-industrial value of about 280 cm^3 m^{-3} to the current level of 391 cm^3 m^{-3}. Therefore, it is necessary to elucidate the role of O_3 and the interaction between O_3 and CO_2 on photosynthesis as a basis of crop dry-matter production under changing climatic conditions. This study was conducted to ascertain the effects of acute O_3 exposure and elevated CO_2 on rice plant physiology such as photosynthetic activity and related characteristics (leaf thickness and inclination angle) and leaf components (antioxidants and plant hormones). The following results were obtained.

(1) The maximum quantum efficiency of PSII (F_v/F_m) in rice leaves exposed to different O_3 concentrations (0, 0.1, and 0.3 cm^3 m^{-3}, expressed as O^0, $O^{0.1}$, and $O^{0.3}$) under different dark adaptation periods (0, 1, 5, 10, 20, and 30 min, expressed as D^0, D^1, D^5, D^{10}, D^{20}, and D^{30}) was examined to ascertain its optimum time span. The F_v/F_m was inhibited by O_3. However in the O^0 and $O^{0.1}$ plants, it recovered during dark adaptation. In the $O^{0.3}$ plants, F_v/F_m decreased gradually with time. The F_v/F_m ratio in the O^0 plants was fully recovered at D^{10}. The effects of O_3 would partially disappear when the dark adaptation period was greater than 20 min. However, it was difficult to distinguish the effects of O_3 and other factors (e.g., light) before D^5. These results demonstrate that the optimum dark adaptation period in rice leaves is 10 min because the effect of O_3 remains maximal, whereas the effects of other factors on F_v/F_m disappear during this period.

(2) To elucidate the interactive effects of O_3 and CO_2 on photosynthesis in rice leaves, experiments of four kinds were conducted: (a) gas exchange, chlorophyll fluorescence, ascorbic acid and glutathione were examined under acute, combined exposures of O_3 (O^0,

$O^{0.1}$, and $O^{0.3}$), and CO_2 (400 or 800 cm^3 m^{-3}, expressed as C^{400} or C^{800}, respectively). The P_N and PSII activity in young (8^{th}) leaves were inhibited during O_3 exposure. However, these were ameliorated by C^{800}. They fully recovered within 3 d except in $O^{0.3}$ + C^{400} plants. The O_3-inhibition of photosynthesis in the flag leaves at anthesis was smaller and the recovery was faster than that of the 8^{th} leaves. Elevated CO_2 interactively ameliorated the O_3-inhibition of photosynthesis and antioxidants levels in flag leaves were higher than those in 8^{th} leaves. Consequently, the predicted differences of O_3-inhibition of photosynthesis between leaf positions were ascribable to their respective leaf thickness and leaf inclination angle. The following two experiments were conducted respectively in relation to inclination angle and antioxidant level. (b) The middle portions of the flag leaves were set horizontally using lead weights (H plant) and were compared with those of a non-treated, erect leaf (E plant). The O_3-inhibition of photosynthesis on the H plant was severer than that on the E plant. (c) The effect of exogenous ascorbic acid on O_3-inhibition of photosynthesis was examined. The O_3-inhibition of photosynthesis in $O^{0.1}$ plants was ameliorated by ascorbic acid application, although those in $O^{0.3}$ plants were unaffected. In addition, total ascorbic acid contents increased by ascorbic acid application. These results show that the leaf thickness (antioxidant levels) and leaf inclination angle are determinants for O_3-inhibition of photosynthesis. (d) 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. The effect of acute exposure to O_3 on leaf photosynthesis of paddy rice grown under different CO_2 concentration was examined. Photosynthesis was inhibited immediately after O_3 exposure, but these were ameliorated by C^{800}, as in the case of short CO_2 treatment. The acclimation of g_s to elevated CO_2 occurred in rice leaves of C^{800} plants. However, no significant difference was found between C^{800} + $O^{0.3}$ plants and C^{400} $\rightarrow C^{800}$ + $O^{0.3}$ plants (grown at C^{400} + O^0 and received acute C^{800} + $O^{0.3}$ exposure). The total ascorbic acid contents

per fresh and dry weight bases decreased by C^{800} because the specific leaf weight (SLW) increased significantly by C^{800}. Therefore, long-term exposure of rice plants to elevated CO_2 might have little effect on antioxidant systems.

(3) Nitrogen metabolism is particularly important for plant growth because it regulates photosynthesis and the C/N ratio. Therefore, the interaction between O_3 and CO_2 on photosynthesis and nitrogen metabolism in rice was evaluated. The PSII and nitrite reductase (NiR) activity were inhibited by O_3, but they were ameliorated by elevated CO_2. This result revealed that the O_3-inhibition of photosynthesis accompanies the reduced electron transport toward the downstream of photosystem and suggests that the reduction power became deficient.

(4) To ascertain the role of plant hormone on O_3-inhibition of photosynthesis in rice, the effects of exogenous and endogenous jasmonates (JA and MeJA), salicylic acid (SA) and ethylene were exanimed. The MeJA ameliorated the O_3-inhibition of PSII. Rapid recovery from O_3-inhibition of P_N was observed after MeJA application, but MeJA degraded the gas exchange. The MeJA application increased the total ascorbic acid content. With respect to endogenous JA and MeJA, MeJA contents were increased immediately after O_3 exposure, and thereafter decreased, although JA contents were increased with a lag time. The SA ameliorated the O_3-inhibition of P_N slightly, and ameliorated the O_3-induced depletion of total ascorbic acid and RDS substantially. With respect to endogenous SA, O_3 exposure elevated the level of endogenous SA. The ethylene action inhibitor (STS) application ameliorated the O_3-induced visible leaf symptom and O_3-inhibition of photosynthesis, but ethephon application slightly worsened or did not affect them. The O_3 exposure elevated the ethylene production from leaves immediately after the exposure and thereafter decreased rapidly. These results indicate that JA and SA act as negative regulators of O_3 inhibition, and that ethylene acts as a positive regulator. Because the increment of SA in this study is smaller than in *Arabidopsis*, SA might act in downstream of JA and ethylene. Moreover, the role of

SA might be minor in rice leaves compared to those of other plants. Both JA and ethylene might be major determinants for O_3 injury in rice leaves.

According to the results and discussion presented up to this point, the author infers the following. (1) O_3-inhibition of rice photosynthesis is ameliorated interactively by elevated CO_2 mainly throught stomatal closure. (2) The antioxidant system and related hormone biosynthesis are induced. However, rice plants are unable to overcome damages induced by O_3 at high O_3 concentrations such as 0.3 cm^{-3} m^{-3}. (3) The application of antioxidative chemicals for protection from O_3 damage benefits paddy rice production.

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* In Japanese.

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