O_2^- Activates Leaf Injury, Ethylene and Salicylic Acid Synthesis, and the Expression of O_3-Induced Genes in O_3-Exposed Tobacco

Daisuke Ogawa[a,b], Nobuyoshi Nakajima[b,*], Masanori Tamaoki[b], Mitsuko Aono[c], Akihiro Kubo[c], Hiroshi Kamada[a], and Hikaru Saji[c]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[b] Biodiversity Conservation Research Project, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan. Fax: +81-29-850-2490. E-mail: naka-320@nies.go.jp
[c] Environmental Biology Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

* Author for correspondence and reprint requests

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O_3 is the major component of photochemical oxidants and gives rise to visible injuries on plant leaves. In O_3-exposed plants, O_2^- is produced before the formation of the injury, but the role that O_2^- plays in plant response to O_3 exposure is still unknown. To clarify its role, we observed the behavior of plants during O_3 exposure after pretreatment with tiron, which is an O_2^- scavenger. When tiron-pretreated tobacco cv. Bel W3 was exposed to O_3, leaf damage was attenuated. In O_3-exposed tobacco, tiron inhibited increases in the levels of ethylene and salicylic acid, which promote leaf injury. Tiron pretreatment also suppressed increases in the expression of O_3-induced genes. These results suggest that O_2^- is involved in many plant responses induced by O_3 exposure. Bel B, a tobacco cultivar that is genetically related to Bel W3, is reported to be more resistant to O_3 than Bel W3, but the reason for this difference is unclear. We investigated the differences between the responses of Bel B and tiron-pretreated Bel W3 to O_3 exposure, and we discuss the reasons for the resistance to O_3 by comparing the phenotype of Bel B with that of tiron-pretreated Bel W3.

Key words: Ozone, Superoxide Radical, Tiron

Introduction

Ozone (O_3) is the most prevalent photochemical oxidant in the atmosphere, and it reduces the growth and productivity of agricultural crops and forest trees (Preston and Tingey, 1988). Rao *et al.* (2000a) proposed that plant leaves absorb O_3 through the stomata and that the incorporated O_3 generates reactive oxygen species such as the superoxide anion radical (O_2^-). O_2^- is produced during exposure to a wide range of environmental stresses, such as drought, high salinity, and low temperature (Bowler *et al.*, 1992; Noctor and Foyer, 1998; Desikan *et al.*, 2001; Allen, 1995). The production of O_2^- during exposure to these stresses results from photorespiration, photosynthesis, and mitochondrial respiration. In addition, pathogen infection, wounding, drought, and osmotic stress have been shown to trigger the explosive production of O_2^- by means of activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which catalyze the conversion of O_2 to O_2^- on the cell membrane. Loss of *AtrbohD* and *AtrbohF*, genes that encode NADPH oxidase in *Arabidopsis*, leads to impaired abscisic acid-induced stomatal closing in guard cells, which suggests that O_2^- is required for stomatal closure (Kwak *et al.*, 2003). Moreover, when plants are infected with a pathogen, cell death is induced to prevent pathogen proliferation. Knockdown of *Nbrboh* genes that encode *NADPH oxidase* in tobacco reduces and delays cell death triggered by treatment with an elicitor (Yoshioka *et al.,* 2003). This evidence indicates that O_2^- plays an important role in plant response to environmental stresses.

O_3-exposed plants have been reported to synthesize phytohormones, including ethylene and salicylic acid (SA) (Rao *et al.*, 2000b; Rao and Davis, 2001; Overmyer *et al.*, 2003). Ethylene has been implicated in developmental processes such as formation of the apical hook in dark-grown

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seedlings, regulation of cell expansion, senescence, abscission, and fruit ripening (Johnson and Ecker, 1998; Wang *et al.*, 2002; Chen *et al.*, 2005). Ethylene is synthesized from L-methionine via *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylate (ACC), and ACC synthase (ACS) is the rate-limiting enzyme for ethylene synthesis. When the activity of ACS is inhibited by aminoethoxyvinylglycine or by suppression of *ACS* expression in the *ACS* anti-sense line, O_3-induced ethylene synthesis decreases and the magnitude of leaf damage is moderated (Mehlhorn and Wellburn, 1987; Nakajima *et al.*, 2002). Furthermore, the propagation of leaf injury in O_3-exposed plants is also attenuated by treatment of the plants with an inhibitor of ethylene signaling, such as norbornadiene or 1-methylcyclopropene (1-MCP) (Bae *et al.*, 1996; Tamaoki *et al.*, 2003). Thus, O_3-induced ethylene production enhances leaf damage in plants.

SA is a key endogenous molecule that mediates disease resistance in tobacco and *Arabidopsis*, and thermogenesis of the appendix in voodoo lilies (Malamy *et al.*, 1990; Gaffney *et al.*, 1993; Raskin *et al.*, 1987). When plants (including tobacco, *Arabidopsis,* and poplar) are exposed to O_3, accumulation of SA is observed (Yalpani *et al.*, 1994; Sharma *et al.*, 1996; Koch *et al.,* 2000). In tobacco, SA is synthesized via phenylalanine (Ogawa *et al.*, 2005). The role of SA in O_3-exposed plants has been studied by analysis of a transgenic plant into which researchers introduced a bacterial SA hydroxylase encoding an enzyme that catalyzes the conversion of SA into catechol. The transgenic plant could not accumulate O_3-induced SA in the leaves and showed reduction of leaf injury by O_3, which indicates that SA enhances O_3-induced leaf damage in tobacco and *Arabidopsis* (Örvar *et al.*, 1997; Rao and Davis, 1999).

O_2^- is known to accumulate in O_3-exposed plants, and diphenylene iodonium, an inhibitor of NADPH oxidase, inhibits the increase in leaf damage by O_3 (Overmyer *et al.* 2000); these results imply that O_2^- may play an important role in the generation of leaf injury in O_3-exposed plants. However, the details regarding the role of O_2^- are still unknown. It is known that 1,2-dihydroxy-3,5-benzenedisulfonic acid (tiron) reacts with O_2^-. Tiron has been used as a probe for O_2^- formation in biological systems (Greenstock and Miller, 1975). In tomato, tiron pretreatment inhibits O_3-induced ethane emission, which is an indicator of leaf injury (Bae *et al.,* 1996). This result suggests that tiron may be a useful O_2^- scavenger. Here, we investigated the generation of leaf injury, and we measured ethylene and SA levels and the expression of O_3-induced genes during O_3 exposure in tiron-pretreated tobacco cv. Bel W3 to clarify the role of O_2^- in O_3-exposed tobacco.

Bel W3 is an O_3-sensitive tobacco cultivar, whereas genetically related tobacco cv. Bel B is resistant to O_3 (Heggestad, 1991). Moreover, Bel B accumulates less H_2O_2 after O_3 exposure than does Bel W3 (Schraudner *et al.*, 1998). O_2^- is thought to disproportionate to H_2O_2 and O_2, either spontaneously or by the activity of superoxide dismutase (SOD) (Rao *et al.*, 2000a). Therefore, during O_3 exposure, the O_3-resistant phenotype of Bel B may depend on the inhibition of O_2^- generation and propagation. To investigate this possibility, we compared the responses of Bel B and tiron-pretreated Bel W3 to O_3.

Results

Formation of leaf injury during O_3 exposure was attenuated by tiron pretreatment

To determine the role of O_2^- generated from O_3 in plants *in vivo*, we investigated the phenotype of O_3-exposed plants with and without pretreatment with tiron. The leaf damage covered more than 80% of the leaf area when untreated tobacco was exposed to O_3, whereas pretreatment with tiron significantly inhibited the formation of leaf injury (Figs. 1A and B). These results suggest that O_2^- promotes the formation of leaf injury in O_3-exposed tobacco.

O_3 is absorbed into plant tissues through the stomata. To confirm that tiron does not influence the stomatal aperture, we examined the stomatal conductance in O_3-exposed tobacco with and without tiron pretreatment. The stomatal conductance of both tiron-pretreated and control leaves increased during the first 1 h after the initiation of O_3 exposure, and the conductance decreased until 4 h (Fig. 2). Although the stomatal conductance in tiron-pretreated leaves at 2 h was higher than that in control leaves, the stomatal conductance values at 0, 1, 4, and 6 h were not significantly different for the tiron-pretreated and control leaves. This result suggests that the inhibition of leaf injury in tiron-pretreated leaves was not caused by changes in the absorption rate of O_3.

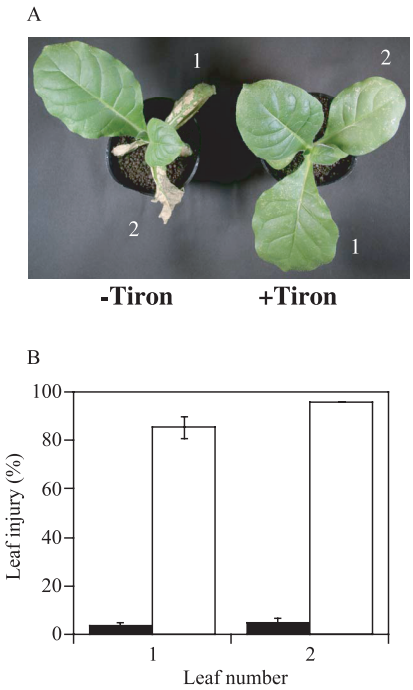

Fig. 1. Leaf injury in O_3-exposed tobacco attenuated by tiron pretreatment. Tobacco plants (4–5 weeks old) were fumigated with 0.2 mL L^{-1} O_3 for 6 h. Tiron pretreatment was performed 2 h before the start of O_3 exposure. (A) Tobacco plants after O_3 exposure for 6 h and incubation in a free-air room for 18 h. Numbers indicate the first and second leaves. (B) Extent of visible injury due to O_3 exposure. Black bar, tiron-pretreated plants; white bar, control plants; vertical bars, standard deviations obtained from three replicates.

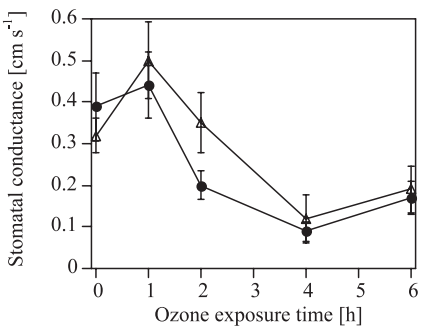

Fig. 2. Stomatal conductance for tiron-pretreated plants and control plants. Four- to five-week-old tobacco plants were fumigated with 0.2 mL L^{-1} O_3 for 6 h. Tiron pretreatment was performed 2 h before the start of O_3 exposure. Vertical bars, standard deviations obtained from three replicates; solid circles, control plants; open triangles, tiron-pretreated plants.

Tiron pretreatment inhibited production of ethylene and SA during O_3 exposure

Ethylene and SA stimulate the propagation of leaf injury in O_3-exposed plants (Mehlhorn and Wellburn, 1987; Nakajima *et al.*, 2002; Örvar *et al.*, 1997). To determine whether O_2^- promotes ethylene and SA synthesis upon O_3 exposure, we measured the levels of these phytohormones in tiron-pretreated tobacco. Ethylene emission by both control and tiron-pretreated tobacco began 2 h after the initiation of O_3 exposure and increased gradually until 6 h (Fig. 3A). However, the level of ethylene evolution in tiron-pretreated tobacco was significantly lower than that in control tobacco during O_3 exposure. In control tobacco, accumulation of SA was detected 4 h after the initiation of O_3 exposure, and the level of SA increased until 6 h (Fig. 3B). By contrast, only a slight increase in SA accumulation was observed in tiron-pretreated tobacco. These results suggest that the production of O_2^- promotes ethylene and SA synthesis during O_3 exposure in tobacco.

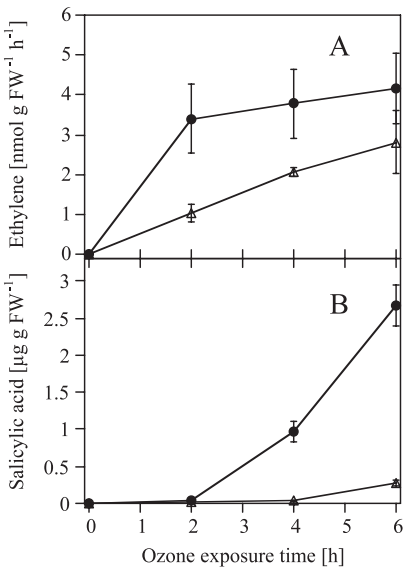

Fig. 3. Increases in (A) ethylene levels and (B) SA levels due to O_3 exposure inhibited by tiron pretreatment. Four- to five-week-old tobacco plants were fumigated with 0.2 mL L^{-1} O_3 for 6 h. Tiron pretreatment was performed 2 h before the start of O_3 exposure. Vertical bars, standard deviations obtained from three replicates; solid circles, control plants; open triangles, tiron-pretreated plants.

Tiron pretreatment suppressed induction of O_3-responsive genes

Previous reports have shown that O_3 exposure in plants induces the expression of genes that encode enzymes for phytohormone and polyamine biosynthesis, proteins for the regulation of redox balance, and pathogenesis-related (PR) proteins (Kubo, 2002). To investigate whether O_2^- contributes to the induction of genes by O_3, we analyzed the levels of O_3-induced expression of genes in control and tiron-pretreated plants by means of Northern blot analysis. O_3 exposure for 2 h more than doubled the expression of *ACS2*, *ACO*, *PAL A*, *GGL1*, *LOX*, *ADC*, *ODC,* and *CuZnSOD*. At 4 h, the expression levels of *APX*, *GPX,* and *GST* were also increased by O_3 exposure. We refer to these 11 genes as O_3-induced genes. The induction of all these O_3-induced genes was inhibited by tiron pretreatment (Table I). This result suggests that O_2^- enhanced the expression levels of O_3-induced genes during O_3 exposure. By contrast, the expressions of *AOS*, *OPR*, *SADC*, *SPDS*, *MnSOD*, *CAT1*, *SACAT,* and *GR* were not increased by O_3 exposure.

The pattern of gene expression in Bel B was similar to that in tiron-pretreated Bel W3

Tobacco Bel B, which is genetically related to Bel W3, also exhibits resistance to O_3 (Heggestad, 1991). To investigate the O_3-tolerant phenotype in tobacco Bel B, we compared leaf injury, hormones levels, and the expression of O_3-induced genes for Bel B and tiron-pretreated Bel W3 during O_3 exposure under our experimental conditions. In response to O_3 exposure, Bel B showed less leaf injury than Bel W3 (data not shown), and ethylene and SA levels in Bel B were significantly lower than in Bel W3 (Figs. 4A and B). Induction rates for *ACS2*, *ACO*, *PAL A*, *GGL1*, *LOX*, *ADC, ODC*, *GPX,* and *GST* were remarkably lower in Bel B than in Bel W3 either at 2 h or at 4 h, or at both times (Table II). However, although at 2 h the induction rates for *CuZnSOD* and *APX* in Bel

Table I. Increases in gene expression rates due to O_3 exposure (2 h and 4 h) for tiron-pretreated Bel W3 and control. Gene expression values were determined by Northern blot analysis. Rate increases were calculated by comparing the signal intensities of O_3-exposed samples to the intensities of samples incubated in a free-air chamber. Control, no tiron pretreatment; +tiron, pretreated with tiron.

Enzyme	2 h		4 h		Number
	Control	+ Tiron	Control	+ Tiron	
ACC synthase 2 (*ACS2*)	17.5	1.1	25.8	8.1	AF057563
ACC oxidase (*ACO*)	5.4	3.7	7.6	3.8	Z29529
Phenylalanine ammonia lyase A (*PAL A*)	15.2	2.2	14.0	2.4	AB008199
β-1,3 Glucanase 1 (*GGL1*)	3.2	1.1	11.2	2.2	AF141653
Lipoxygenase (*LOX*)	33.9	3.6	19.2	9.7	AB233415
Allen oxide synthase (*AOS*)	0.4	0.9	0.5	0.7	AB233414
12-Oxo-phytodienoic acid reductase (*OPR*)	0.6	1.5	0.5	1.6	AB233416
S-Adenosylmethionine decarboxylase (*SADC*)	1.2	1.3	1.1	1.3	U91924
Arginine decarboxylase (*ADC*)	6.6	3.5	8.2	2.8	AB110952
Ornithine decarboxylase (*ODC*)	54.0	11.0	211.5	20.0	AB021066
Spermidine synthase (*SPDS*)	0.9	0.7	1.0	1.3	AB006692
Mn superoxide dismutase (*MnSOD*)	0.9	1.1	1.5	3.6	X14482
Cu/Zn superoxide dismutase (*CuZnSOD*)	2.3	1.3	6.2	1.7	X55974
Catalase 1 (*CAT1*)	1.0	1.3	0.5	0.6	U93244
Salicylic acid-binding catalase (*SACAT*)	0.3	0.6	0.8	1.5	U03473
Ascorbate peroxidase (*APX*)	1.5	1.6	2.2	1.6	U15933
Glutathione peroxidase (*GPX*)	1.4	1.0	4.0	1.0	D10524
Glutathione reductase (*GR*)	0.9	1.4	1.0	0.7	X76533
Glutathione S-transferase (*GST*)	1.3	1.3	11.7	3.2	D10524

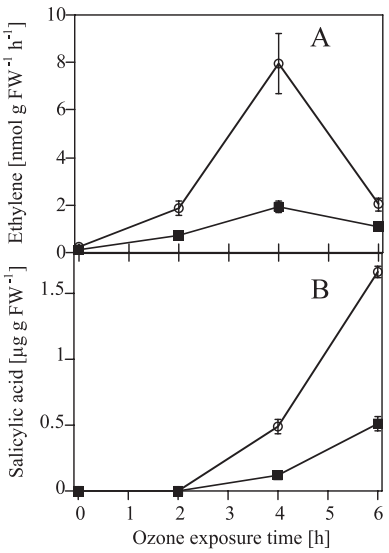

Fig. 4. (A) Ethylene levels and (B) SA levels in O_3-exposed Bel B were lower than those in Bel W3. Four- to five-week-old tobacco plants were fumigated with 0.2 mL L^{-1} O_3 for 6 h. Vertical bars, standard deviations obtained from three replicates; open circles, Bel W3; solid squares, Bel B.

B were slightly lower than those for Bel W3, the rates for Bel B at 4 h were higher than those for Bel W3. These results indicate that the induction levels of many O_3-responsive genes in Bel B were lower than those in Bel W3. Taken together, our results show that Bel B and tiron-pretreated Bel W3 respond similarly to O_3 exposure.

Discussion

Pretreatment of Bel W3 tobacco with tiron dramatically inhibited O_3-induced leaf injury (Figs. 1A and B). This result suggests that O_2^- induced the leaf injury in the O_3-exposed plants. In *Arabidopsis*, O_2^- produced by the xanthine/xanthine oxidase system induces an increase in ion leakage from leaves, which is an indicator of leaf injury (Overmyer *et al.*, 2000). This result supports the hypothesis that O_2^- has the ability to cause leaf injury during O_3 exposure.

Ethylene and SA have been reported to promote leaf damage in O_3-exposed plants (Mehlhorn and Wellburn, 1987; Nakajima *et al.*, 2002; Örvar *et al.*, 1997). In tiron-pretreated tobacco, increases in the levels of these hormones during O_3 exposure were suppressed (Fig. 3A and B). These results imply that O_2^- enhances increases in the levels of these hormones in O_3-exposed tobacco. The dramatic inhibition of leaf injury during O_3 exposure in tiron-pretreated tobacco may be attributable not only to dismutation of O_2^- but also to decreases in ethylene and SA levels.

The activation of O_3-induced genes, such as *ACS2*, *ACO*, *PAL A*, *GGL1*, *LOX*, *ADC*, *ODC*, *CuZnSOD*, *APX*, *GPX,* and *GST,* was suppressed by tiron pretreatment (Table I). This result implies that O_2^- activates the expression of these genes during O_3 exposure. It is known that *ACS2* and *ACO* are important enzymes for the biosynthesis of ethylene, and that *PAL* is important for the biosynthesis of SA (Kende, 1993; Ogawa *et al.*, 2005). However, which gene serves for these phytohormones synthesis remains to be determined. In our

Enzyme	2 h		4 h	
	Bel W3	Bel B	Bel W3	Bel B
ACS2	10.2	6.2	7.5	4.8
ACO	2.9	2.8	3.1	1.9
PAL A	18.9	4.4	30.8	17.2
GGL 1	12.2	10.8	72.7	34.2
LOX	54.5	11.0	33.5	31.6
ADC	8.9	1.7	7.7	7.3
ODC	13.4	6.3	239.0	260.0
CuZnSOD	1.5	1.0	2.9	3.8
APX	1.9	0.6	1.6	3.0
GPX	1.1	1.0	12.7	5.1
GST	1.3	1.4	34.7	10.0

Table II. Increases in gene expression rates due to O_3 exposure (2 h and 4 h) in Bel W3 and Bel B tobacco. Gene expression values were determined by Northern blot analysis. Rate increases were calculated by comparing the signal intensities of O_3-exposed samples with the intensities of samples incubated in a free-air chamber.

experiments, the expression levels of *ACS2*, *ACO,* and *PAL A* were increased by O_3 exposure, and the increases were inhibited by pretreatment with tiron (Table I). We showed that ethylene emission and SA accumulation were induced in O_3-exposed tobacco and that the production of these compounds was suppressed by tiron pretreatment (Figs. 3A and B). These results show that the pattern of gene expression was associated with the levels of these hormones. Accordingly, it is highly likely that these genes regulate these hormones biosyntheses in O_3-exposed tobacco.

Bel B is known to be more resistant to O_3 than Bel W3 (Heggestad, 1991). However, the reason for the increased resistance has not been determined. Therefore, we compared the behavior of Bel B with that of tiron-pretreated Bel W3. The levels of ethylene emission and SA accumulation in Bel B were lower than those in Bel W3 (Figs. 4A and B), as reported previously (Schraudner *et al.*, 1998; Pasqualini *et al.*, 2002). During O_3 exposure, the induction of O_3-induced genes, such as *ACS2*, *ACO*, *PAL A*, *GGL1*, *LOX*, *ADC*, *ODC*, *GPX,* and *GST*, was suppressed in Bel B compared with Bel W3 (Table II). The increases in ethylene and SA levels and in the expression levels of these genes during O_3 exposure were also inhibited in tiron-pretreated tobacco (Figs. 3A and B and Table I). Therefore, these results indicate that the responses to O_3 in Bel B were similar to those in tiron-pretreated Bel W3. Bel B accumulates less H_2O_2 after O_3 exposure than does Bel W3, and O_2^- disproportionates to H_2O_2 and O_2 in plant tissue (Schraudner *et al.*, 1998). Taken together the results of Schraudner *et al.* and our results suggest that the O_3 tolerance of Bel B may be due to the presence of low levels of O_2^- during O_3 exposure. In fact, at 4 h, Bel B showed higher induction rates for *CuZnSOD* and *APX*, which encode redox enzymes, than did Bel W3 (Table II). This phenomenon may be a part of the system responsible for inhibiting the generation of O_2^-.

Bel W3 shows enhanced H_2O_2 accumulation, high levels of ethylene and SA, and high expression levels of O_3-induced genes during O_3 exposure (Schraudner *et al.,* 1998; Pasqualini *et al.,* 2002). An O_3-sensitive phenotype has been observed in the *Arabidopsis* mutant *rcd1*, and production of O_2^-, ethylene, and SA in *rcd1* during O_3 exposure increases more in the mutant that in the wild type (Overmyer *et al.*, 2000, 2005). On the basis of these reports, we can presume that O_2^- is involved in these responses. However, because the genes responsible for the sensitive phenotype in Bel W3 have not been found and function of *RCD1* remains to be clarified, the degree of O_2^- participation in O_3-induced responses has not been clarified. We succeeded in scavenging O_2^- by using tiron *in vivo*, and we have provided more detailed information about the role of O_2^- in O_3-exposed plants.

Materials and Methods

Plant materials and O_3 fumigation

Tobacco seeds (*Nicotiana tabacum* L. cv. Bel W3 or Bel B) were germinated on culture soil (Kureha Chemical Industry Co., Tokyo, Japan), and seedlings were grown in a controlled-environment greenhouse at 25 °C during the day and at 20 °C at night, with a relative humidity of 70% and a 14-h light/10-h dark cycle. Plants were watered daily. The fully developed leaf at the highest position is referred to as the first leaf (Fig. 1A).

We exposed 4- to 5-week-old plants in a chamber to a single dose of O_3 (0.2 μL L^{-1}) produced with an O_3 generator (Sumitomo Seika Chemicals, Osaka, Japan). O_3 fumigation was carried out at 25 °C at a relative humidity of 70% under a photosynthetic photon flux density (PPFD) of 200 μmol photons m^{-2} s^{-1} in continuous light.

Treatment of tiron

We sprayed tobacco plants with 10 mM tiron solution containing 0.1% Tween 20. We used plants sprayed with 0.1% Tween 20 as control plants. Both plants underwent vacuum infiltration. These plants were used for experiments 2 h after pretreatment with tiron.

Extent of leaf injury

The extent of leaf injury was measured as described by Nakajima *et al.* (2002). Plants were exposed to O_3 for 6 h and then transferred to a fresh-air chamber in continuous light. 24 h after the start of O_3 exposure, the first, second, and third leaves were excised and scanned (GT7600U; Epson Tokyo, Japan) into a computer. The area of visible damage on each leaf was calculated by using image-analysis software (NIH Image; National Institutes of Health, Washington, DC, USA).

Measurement of ethylene production

Ethylene production was determined by means of a procedure reported by Bae *et al.* (1996), with minor modifications. At sampling time, the first and second leaves were removed from the plants and incubated in sealed 100-mL flasks under light for 1 h. Then 1 mL of gas was withdrawn from the flasks, and the ethylene content was analyzed with a gas chromatograph equipped with a flame ionization detector (GC-7 A; Shimadzu, Osaka, Japan).

SA extraction and quantification

Salicylic acid was extracted from 0.5 g of tobacco leaves. Each sample was extracted four times with 1.5 mL methanol. We added 5 μL of 1 mg mL^{-1} *m*-hydroxybenzoic acid as an internal standard. After the extracts were combined and evaporated to dryness, the residue was dissolved in 100 μL methanol, and 600 μL of 1 mM KOH were added. Lipophilic substances were removed by extraction twice with chloroform. The aqueous phase was transferred to a new tube, and 10 μL of phosphoric acid and 700 μL of ethyl acetate were added. The solution was mixed and centrifuged at $17{,}000 \times g$ for 10 min. The supernatant was transferred to a new tube and again extracted with ethyl acetate. The supernatants were combined and evaporated to dryness, and the residue was dissolved in 50% methanol and analyzed by HPLC (System Gold; Beckman, CA, USA). SA was detected with a fluorescent detector (RF-530; Shimadzu, Osaka, Japan) (excitation wavelength, 295 nm; emission, 370 nm). The mobile phase was 20 mM sodium acetate (pH 2.5) containing 20% methanol.

Preparation of cDNA probes

Complementary DNA (cDNA) of genes in tobacco was isolated by reverse transcription polymerase chain reaction (RT-PCR) using total RNA obtained from O_3-exposed tobacco. The following genes were cloned: *ACS2* and *ACO*, encoding enzymes for ethylene biosynthesis; *PAL A*, encoding enzyme for SA biosynthesis; *GGL1*, SA-induced PR protein; *LOX*, *AOS* and *OPR*, encoding enzymes for jasmonic acid biosynthesis; *MnSOD*, *CuZnSOD*, *CAT1*, *SACAT*, *APX*, *GPX*, *GR,* and *GST*, encoding enzymes for redox regulation; and *SADC*, *ADC*, *ODC,* and *SPDS*, encoding enzymes for polyamine biosynthesis. The standard nomenclature is shown in Table I.

The primers for RT-PCR were designed according to the published cDNA sequences for various plants (*MnSOD*, 5′-GCACTACGAACCCTAGTGAGCAGAC-3′ and 5′-TCCATATGTTCTTCAGATAATCAGG-3′; *CuZnSOD*, 5′-GTGAAGGCCGTTGCCGTCCTTAGCA-3′ and 5′-TGGAGGCCGATGATACCACAAGCAAC-3′; *APX*, 5′-CTCAGAGGACTCATTGCTGAGAAGA-3′ and 5′-ACTTCAGCAAATCCCAACTCAGAGA-3′; *GST*, 5′-CCATGTCAACTGCAACCATGAGAGT-3′ and 5′-ATATCAGCACACCATGCACTCACAC-3′; *CAT1*, 5′-CATACAAGTACCGTCCGTCAAGTG-3′ and 5′-GAGTCATAGAGGTCTTGAGTAGCGTG-3′; *SADC*, 5′-AAGATCATTTGTTGCTGTGA-3′ and 5′-AAGAGAGTAGCCTTTCACAT-3′; *ADC*, 5′-CGACCAAGTCAAGGTCGAGCT-3′ and 5′-GTCAGAGATGGCAACGGGAC-3′; *GR*, 5′-CTGAAGAATGCTGGTGTCACTCT-3′ and 5′-ACACATATGCAAGCCCAGAACT-3′; *SPDS*, 5′-AGATGAGTTTATCTACCATGA-3′ and 5′-GATGGTTTTGTTGAGAGTAG-3′; *ODC*, 5′-GCCATTCTTCAGTCCACAAT-3′ and 5′-TCGTAAAGTACACAGTTCATCG-3′; *SACAT*, 5′-GTTATTTGCCGTTTCTCTACTG-3′ and 5′-TGATGAGCACACTTGGGAGCATT-3′; *GPX*, 5′-GCCAGCCAATCTAGCAAGCCTCAATC-3′ and 5′-TCTTGATATCCTTCTCCATGCTAGC-3′; *PAL A*, 5′-AGTTCTCTCAGCTATTTTTGCTG-3′ and 5′-GTTCTCCATTGGTACCCATTGT-3′; *GGL1*, 5′-TCAAAGCTCTCAA-TGGAAGTAAC-3′ and 5′-CAATAGTTGCTG-CAGAGCTTCC-3′; *ACO*, 5′-TT-(T,C)GGIACIA-A(A,G)GT(T,G)AG(T,C)AA-(T,C)T(A,T)(A,T)-CC(A,G)-3′ and 5′-(T,C)TTCATIGC(T,C)TC(A,G)AA(T,G)C(T,G)(T,C)GGCTCCTT-3′; *ACS2*, 5′-ATICA(A,G)ATGGGI-(T,C)TIGCIGA(A,G)-AA(T,C)CA-3′ and 5′-GTICCIA(A,G)TTIGA-IGG(A,G)TT-3′; *LOX*, 5′-AAAGGGACAGTG-GTGTTGATG-3′ and 5′-CATCAGTCATCCAT-GCAGAC-3′; *OPR*, 5′-CTGATCACTGAAGG-CACTATG-3′ and 5′-ACGAGATCAGCATCA-CCTTGT-3′; *AOS*, 5′-TTGAAAAGAAGGAT-CTCTTCAC-3′ and 5′-CAGCACACTGTTTAT-TCTCCAC-3′). The amplified cDNAs were subcloned into a pGEM-T Easy system (Promega, MO, USA) and sequenced with an ALFred sequencer (Amersham Biosciences, NJ, USA).

RNA gel blot analysis

Total RNA from leaves was extracted by means of the sodium dodecyl sulphate (SDS)–phenol method described by Ogawa *et al.* (2005). Total RNA was separated by electrophoresis through a 1.2% agarose gel containing 1.8% formaldehyde and was then transferred to a nylon membrane (Hybond N^+; American Biosciences, NJ, USA). Prehybridization and hybridization were performed as described by Tamaoki *et al.* (2003). The probe was prepared by using the MultiPrime labeling system (American Biosciences) with ^{32}P-dCTP (12 MBq mol^{-1}). The filter was washed at 50 °C with 2 × SSC containing 0.1% SDS, and then at 55 °C with 0.2 × SSC containing 0.1% SDS. The filter was exposed to a Bio-Imaging Plate (Fuji Film Co., Kanagawa, Japan), and signals were assessed using a bioimaging analyzer (BAS2000; Fuji Film Co.).

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