O₃ activates leaf injury, ethylene and salicylic acid synthesis, and the expression of O₃-induced genes in O₃-exposed tobacco

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O₃ is the major component of photochemical oxidants and gives rise to visible injuries on plant leaves. In O₃-exposed plants, O₂− is produced before the formation of the injury, but the role that O₂− plays in plant response to O₃ exposure is still unknown. To clarify its role, we observed the behavior of plants during O₃ exposure after pretreatment with tiron, which is an O₂− scavenger. When tiron-pretreated tobacco cv. Bel W3 was exposed to O₃, leaf damage was attenuated. In O₃-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 O₃-induced genes. These results suggest that O₂− is involved in many plant responses induced by O₃ exposure. Bel B, a tobacco cultivar that is genetically related to Bel W3, is reported to be more resistant to O₃ 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 O₃ exposure, and we discuss the reasons for the resistance to O₃ by comparing the phenotype of Bel B with that of tiron-pretreated Bel W3.

Key words: Ozone, Superoxide Radical, Tiron

Introduction

Ozone (O₃) 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 O₃ through the stomata and that the incorporated O₃ generates reactive oxygen species such as the superoxide anion radical (O₂−). O₂− 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 O₂− during exposure to these stresses results from photospiration, photosynthesis, and mitochondrial respiration. In addition, pathogen infection, wounding, drought, and osmotic stress have been shown to trigger the explosive production of O₂− by means of activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxides, which catalyze the conversion of O₂ to O₂− 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 O₂− 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 O₂− plays an important role in plant response to environmental stresses.

O₃-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...
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, O₃-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 O₃-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, O₃-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 O₃, 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 O₃-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 O₃-induced SA in the leaves and showed reduction of leaf injury by O₃, which indicates that SA enhances O₃-induced leaf damage in tobacco and Arabidopsis (Örvar et al., 1997; Raouf and Davis, 1999).

O₃ is known to accumulate in O₃-exposed plants, and diphenylene iodonium, an inhibitor of NADPH oxidase, inhibits the increase in leaf damage by O₃ (Overmyer et al. 2000); these results imply that O₂ may play an important role in the generation of leaf injury in O₃-exposed plants. However, the details regarding the role of O₂ are still unknown. It is known that 1,2-dihydroxy-3,5-benzenedisulfonic acid (tiron) reacts with O₂. Tiron has been used as a probe for O₂ formation in biological systems (Greenstock and Miller, 1975). In tomato, tiron pretreatment inhibits O₂-induced ethane emission, which is an indicator of leaf injury (Bae et al., 1996). This result suggests that tiron may be a useful O₂ scavenger. Here, we investigated the generation of leaf injury, and we measured ethylene and SA levels and the expression of O₃-induced genes during O₃ exposure in tiron-pretreated tobacco cv. Bel W3 to clarify the role of O₂ in O₃-exposed tobacco.

Bel W3 is an O₃-sensitive tobacco cultivar, whereas genetically related tobacco cv. Bel B is resistant to O₃ (Heggestad, 1991). Moreover, Bel B accumulates less H₂O₂ after O₃ exposure than does Bel W3 (Schraudner et al., 1998). O₂ is thought to disproportionate to H₂O₂ and O₂, either spontaneously or by the activity of superoxide dismutase (SOD) (Rao et al., 2000a). Therefore, during O₃ exposure, the O₃-resistant phenotype of Bel B may depend on the inhibition of O₂ generation and propagation. To investigate this possibility, we compared the responses of Bel B and tiron-pretreated Bel W3 to O₃.

Results

Formation of leaf injury during O₃ exposure was attenuated by tiron pretreatment

To determine the role of O₂ generated from O₃ in plants in vivo, we investigated the phenotype of O₃-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 O₃, whereas pretreatment with tiron significantly inhibited the formation of leaf injury (Figs. 1A and B). These results suggest that O₂ promotes the formation of leaf injury in O₃-exposed tobacco.

O₃ is absorbed into plant tissues through the stomata. To confirm that tiron does not influence the stomatal aperture, we examined the stomatal conductance in O₃-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 O₃ 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 O₃.
Tiron pretreatment inhibited production of ethylene and SA during O₃ exposure

Ethylene and SA stimulate the propagation of leaf injury in O₃-exposed plants (Mehlhorn and Wellburn, 1987; Nakajima et al., 2002; Örvar et al., 1997). To determine whether O₂ promotes ethylene and SA synthesis upon O₃ 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 O₃ 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 O₃ exposure. In control tobacco, accumulation of SA was detected 4 h after the initiation of O₃ 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 O₂ promotes ethylene and SA synthesis during O₃ exposure in tobacco.
Tiron pretreatment suppressed induction of O₃-responsive genes

Previous reports have shown that O₃ 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 O₂ contributes to the induction of genes by O₃, we analyzed the levels of O₃-induced expression of genes in control and tiron-pretreated plants by means of Northern blot analysis. O₃ exposure for 2 h more than doubled the expression of ACS2, ACO, PAL A, GGLI, LOX, ADC, ODC, and CuZnSOD. At 4 h, the expression levels of APX, GPX, and GST were also increased by O₃ exposure. We refer to these 11 genes as O₃-induced genes. The induction of all these O₃-induced genes was inhibited by tiron pretreatment (Table I). This result suggests that O₂ enhanced the expression levels of O₃-induced genes during O₃ exposure. By contrast, the expressions of AOS, OPR, SADC, SPDS, MnSOD, CATI, SACAT, and GR were not increased by O₃ 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 O₃ (Heggestad, 1991). To investigate the O₃-tolerant phenotype in tobacco Bel B, we compared leaf injury, hormones levels, and the expression of O₃-induced genes for Bel B and tiron-pretreated Bel W3 during O₃ exposure under our experimental conditions. In response to O₃ 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, GGLI, 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
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 O₃-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 O₃ exposure.

Table II. Increases in gene expression rates due to O₃ 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 O₃-exposed samples with the intensities of samples incubated in a free-air chamber.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>2 h</th>
<th>4 h</th>
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<tbody>
<tr>
<td></td>
<td>Bel W3</td>
<td>Bel B</td>
</tr>
<tr>
<td>ACS2</td>
<td>10.2</td>
<td>6.2</td>
</tr>
<tr>
<td>ACO</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>PAL A</td>
<td>18.9</td>
<td>4.4</td>
</tr>
<tr>
<td>GGL1</td>
<td>12.2</td>
<td>10.8</td>
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<tr>
<td>LOX</td>
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<td>11.0</td>
</tr>
<tr>
<td>ADC</td>
<td>8.9</td>
<td>1.7</td>
</tr>
<tr>
<td>ODC</td>
<td>13.4</td>
<td>6.3</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>APX</td>
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<td>0.6</td>
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<tr>
<td>GPX</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>GST</td>
<td>1.3</td>
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</tr>
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</table>

Discussion

Pretreatment of Bel W3 tobacco with tiron dramatically inhibited O₃-induced leaf injury (Figs. 1A and B). This result suggests that O₂ induced the leaf injury in the O₃-exposed plants. In Arabidopsis, O₂ 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 O₂ has the ability to cause leaf injury during O₃ exposure.

Ethylene and SA have been reported to promote leaf damage in O₃-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 O₃ exposure were suppressed (Fig. 3A and B). These results imply that O₂ enhances increases in the levels of these hormones in O₃-exposed tobacco. The dramatic inhibition of leaf injury during O₃ exposure in tiron-pretreated tobacco may be attributable not only to dismutation of O₂ but also to decreases in ethylene and SA levels.

The activation of O₃-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 O₂ activates the expression of these genes during O₃ 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
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$_2$O$_2$ after O$_3$ exposure than does Bel W3, and O$_3$ disproportionates to H$_2$O$_2$ 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$_2$O$_2$ 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$_3$, 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 pre-treatment 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 M 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 g 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).
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 32P-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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