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Regulation of Salicylic Acid Synthesis in Ozone-Exposed Tobacco and *Arabidopsis*

By

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K e y w o r d s : Phenylalanine ammonia-lyase, isochorismate synthase, chorismate mutase.

S u m m a r y

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Ozone, a major photochemical oxidant, induces leaf injury. Salicylic acid (SA) is a kind of plant hormone and an important regulator of plant resistance to pathogens. In ozone-exposed plants, SA participates in both the formation of leaf injury and the defense response. In pathogen-infected plants, SA is synthesized via two pathways involving phenylalanine or isochorismate. Biosynthesis of SA in ozone-fumigated plants had not been well defined, so we examined it in tobacco and *Arabidopsis*. Salicylic acid accumulated in tobacco exposed to 0.2 ppm ozone for 6 h. At the same time, phenylalanine ammonia-lyase (PAL) activity, its mRNA level, and the level of chorismate mutase (CM) transcripts increased remarkably, whereas isochorismate synthase (ICS) activity did not increase. These results may suggest that ozone-exposed tobacco synthesized SA via the phenylalanine pathway.

Salicylic acid levels also increased in ozone-exposed *Arabidopsis*, but not in *sid2* (*salicylic acid induction-deficient 2*) mutants, in which *ICS1* is defective. Furthermore, ICS activity and the mRNA level of *ICS1* increased dramatically in wild-type *Arabidopsis* after the start of ozone exposure. These results suggest that ozone-exposed *Arabidopsis* synthesizes SA from isochorismate. Therefore, our results imply that the main pathway of ozone-induced SA biosynthesis differs between tobacco and *Arabidopsis*.

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Introduction

Ozone is the main photochemical oxidant that causes leaf damage in many plant species. Such damage decreases the productivity of crops and forests (PRESTON & TINGEY 1988). Ozone penetrates stomata to induce leaf injury (MEHLHORN & al. 1990, SCHRAUDNER & al. 1998). It reacts with the cell wall and cell membrane to produce reactive oxygen species such as superoxide radicals and hydrogen peroxide, which in turn induce salicylic acid (SA) production (KANGASJÄRVI & al. 1994, RAO & DAVIS 2001). Salicylic acid was produced in response to leaf injury in ozone-exposed plants (RAO & DAVIS 1999, RAO & al. 2000).

The pathway of SA synthesis in plants exposed to pathogens has been identified. Phenylalanine ammonia-lyase (PAL) catalyzes the convertion of phenylalanine to *t*-cinnamic acid (CA). Radio-labeled CA and benzoic acid (BA) were metabolized to SA in tobacco mosaic virus (TMV)-inoculated tobacco leaves (YALPANI & al. 1993, RIBNICKY & al. 1998). Levels of SA in transgenic tobacco plants with epigenetically suppressed PAL expression were lower than in wild-type plants when the plants were inoculated with TMV (PALLAS & al. 1996). The increase of SA promoted by avirulent pathogen infection was reduced by a PAL inhibitor (2-aminoindan-2-phosphonic acid) in *Arabidopsis* (MAUCH-MANI & SULSARENKO 1996). These results show that plants synthesize SA from phenylalanine via CA and BA. However, WILDERMUTH & al. 2001 found a new SA synthesis pathway in pathogen-infected *Arabidopsis* that runs from chorismate via isochorismate. Although the SA synthesis pathways in pathogen-infected leaves are known, the pathway in ozone-stressed plants was not known. To reveal the ozone-inducible SA synthesis pathway, we investigated the transcript levels and activities of enzymes involved in SA synthesis in tobacco and *Arabidopsis*.

Material and Methods

Plant materials and ozone treatment

Tobacco seeds (*Nicotiana tabacum* L. cv. SR-1) were germinated on culture soil (Kureha Chemical Industry Co., Japan), and seedlings were grown in a controlled-environment greenhouse at 25 °C day / 20 °C night with a relative humidity of 70%, a 14-h light / 10-h dark cycle. Plants were watered daily.

We exposed 4- to 5-week-old plants for 6 h in a chamber to a single dose of 200 $nL \cdot L^{-1}$ ozone produced by an ozone generator (Sumitomo Seika Chemicals, Japan). Ozone 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}. Plants remaining in charcoal-filtered air served as the controls. *Arabidopsis thaliana* (L.) Heynh. Plants were grown for 2 weeks as described by KANNA & al. 2003. Condition for ozone treatment was at 25 °C at a relative humidity of 70% under PPFD of 100 μmol photons $m^{-2}s^{-1}$.

Preparation of cDNA probes

Complementary DNAs of *PAL*, *CM*, and *ICS* in tobacco were isolated by reverse transcriptase–polymerase chain reaction (RT-PCR) using total RNA obtained from ozone-exposed tobacco. That of *ICS1* in *Arabidopsis* was isolated by RT-PCR using total RNA from ozone-exposed

Arabidopsis. The primers for RT-PCR were designed according to the published cDNA sequences for various plants (*PAL*, 5'-AGTTCTCTCAGCTATTTTTGCTG-3' and 5'-GTTCTCCATTGGTACCCATTGT-3'; *CM*, 5'-CTTCAATCTAAGGTTGGTAGAT-3' and 5'-TTAGTCAAAGGCATAACCCATTC-3'; *ICS*, 5'-ATGCATATCAGTTCTGTTTGCAA-3' and 5'-CCAGCATACATTCTTCGGTCAAA-3'; *AtICS1*;5'-ATGGCTTCACTTCAATTTTCTTC-3' and 5'-TCAATTAATCGCCTGTAGAGATG-3'). The amplified cDNAs were subcloned into a pGEM-T Easy system (Promega, Madison, WI, USA) and sequenced with an ALFred sequencer (Amersham Biosciences, USA).

RNA gel blot analysis

Total RNA from tobacco leaves was extracted by use of the sodium dodecyl sulfate (SDS)–phenol method as described by NAKAJIMA & al. 1995. Total RNA was separated by electrophoresis through a 1.2% agarose gel that contained 1.8% formaldehyde and then was transferred to nylon membrane (Hybond N^+; Amersham Biosciences). Prehybridization and hybridization were performed as described by NAKAJIMA & al. 2001. The probe was prepared by use of the MultiPrime labeling system (Amersham Biosciences) with ^{32}P-dCTP (12 MBq mol^{-1}). The filter was washed with 2×SSC containing 0.1% SDS at 50 °C. The filter was exposed to a Bio-Imaging Plate (Fuji Film Co., Tokyo, Japan), and signals were assessed using a bioimaging analyzer (BAS2000; Fuji Film Co.).

PAL activity

PAL activity was measured as reported by LEGRAND & al. 1976 with minor modifications. Plant materials were ground with a mortar and pestle in liquid nitrogen. A hundred micrograms of sample was transferred to another mortar with 1 mL of extraction buffer (0.1 M borate buffer, pH 8.8, 5 mM mercaptoethanol). After stirring, the homogenate was centrifuged at 20,000 ×g for 10 min. The supernatant was desalted on Sephadex G-25 (NAP-10 columns, Amersham Biosciences) equilibrated with the extraction buffer. The extraction was performed at 4 ℃.

The incubation mixture contained 500 µL of the desalted solution and 33 µL of 2 mM phenylalanine including 0.0925 MBq of L-[U-^{14}C] phenylalanine. After 1 h incubation at 37 ℃, the reaction was stopped by the addition of 33 µL of 9 N sulfuric acid. Five hundred microliters of toluene was added to the incubation mixture, and *t*-cinnamic acid was extracted to organic phase. The radioactivity in the organic phase was determined by liquid scintillation analyzer (2500TR, PACKARD, USA).

SA extraction and quantification

Salicylic acid was extracted from 0.5 g samples of tobacco leaves and 0.2 g samples of *Arabidopsis*. Each sample was extracted 4 times with 1.5 mL methanol. We added 5 µL of 1 mg·mL^{-1} m-hydroxybenzoic acid as an internal standard. The solution was evaporated to dryness, the residue was dissolved in 200 µL methanol, and then 1 mL 1 mM KOH was added. Lipophilic substances were removed by two extraction with chloroform. The aqueous phase was transferred to a new tube, and then we added 10 µL phosphoric acid and 700 µL ethyl acetate. The solution was mixed and centrifuged at 17,000 ×*g* for 10 min. The supernatant was transferred to a new tube and again extracted with ethyl acetate. All supernatants were evaporated to dryness. The residue was dissolved in 50% methanol and analyzed by high performance liquid chromatography (HPLC) (SYSTEM GOLD, BECKMAN, USA). Salicylic acid was detected with a fluorescence detector (RF-530, Shimadzu, Japan) with excitation at 295 nm and emission at 370 nm. The mobile phase was 20 mM sodium acetate, pH 2.5, containing 20% methanol.

ICS activity

The extraction of ICS and the assay were performed according to the method of POULSEN & al. 1991. Plant materials were ground with a mortar and pestle in liquid nitrogen. We transferred 1.5 g of material into another mortar to which 0.05 g of polyvinylpolypyrrolidone (PVPP) and 2 mL of extraction buffer (0.1 M Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA and 1 mM DTT) were

added. After stirring, the homogenate was centrifuged at 10,000 ×g for 30min. The supernatant was desalted at 4 ℃ on Sephadex G-25 (PD-10columns, Amersham Biosciences) equilibrated with 0.1 M Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA and 1 mM DTT.

The incubation mixture (total volume 0.5mL) for assay contained 250 μL of 0.1M Tris-HCl, pH 7.5, 3 mM Ba-chorismate (Sigma, USA), 15 mM $MgCl_2$ and 250 μL of desalted solution. After 1 h incubation at 30 ℃, the reaction was stopped by the addition of 125 μL of MeOH-second-BuOH. After centrifugation, the supernatant was analyzed with a UV detector (280 nm). The mobile phase was 50 mM phosphate buffer, pH 2.5, containing 35% methanol. Isochorismate was determined by transformed SA (YOUNG & GIBSON 1969). After 1 h incubation of the mixture at 30 ℃, the mixture was kept at 100 ℃ for 10 min, and SA was measured by HPLC as described above.

Protein determination

The level of protein was investigated by using a BCA protein assay kit (PIERCE, USA).

Results and Discussion

There are two SA biosynthesis pathways in plants (Fig. 1). Enzymes of CM and PAL are involved in SA synthesis from chorismate via phenylalanine, and ICS is involved in that from chorismate via isochorismate. We examined which pathway is used in ozone-exposed tobacco and *Arabidopsis*.

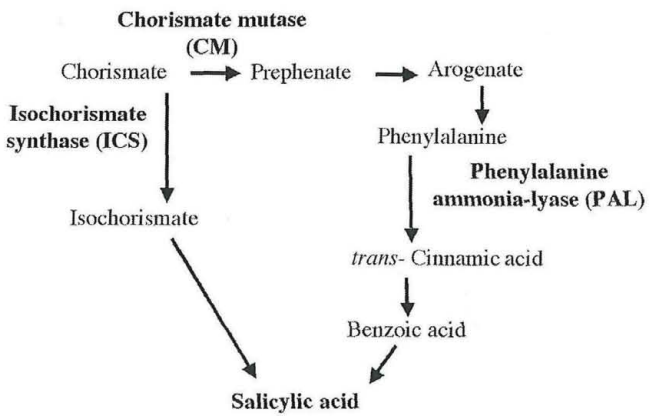

Fig. 1. Proposed pathways of SA biosynthesis.

Salicylic acid accumulated in tobacco exposed to 0.2 ppm ozone for 6 h. The level in ozone-exposed tobacco started to increase by 4 h after the start of ozone exposure and continued to increase to 6 h (Fig. 2a). To determine the levels of transcripts of *CM*, *PAL*, and *ICS*, we isolated cDNAs by RT-PCR of total RNA from ozone-exposed tobacco leaves. The deduced amino acid sequence of the cDNA we isolated for *CM* was 70% identical to that of *Lycopersicon esculentum*, that for *ICS* was 64% identical to that of *Catharanthus roseus*, and that for *PAL* was identical to *PAL A* of tobacco. Therefore, we presumed that the cDNAs we

isolated encoded the respective enzymes. In ozone-exposed tobacco, the level of *CM* transcripts continued to increase for 6 h after the onset of ozone exposure, and that of *PAL A* reached the highest level at 4 h (Figs. 2b, c). On the other hand, that of *ICS* did not increase (data not shown). Furthermore, PAL activity began to increase by 4 h after the start of ozone exposure and reached the highest level at 6 h (Fig. 2d), whereas ICS activity did not increase (data not shown). These results suggest that SA is synthesized from the pathway via phenylalanine. PASQUALINI & al. 2002 reported that, in tobacco, ozone increased the activity of benzoic acid 2-hydroxylase, which catalyzes benzoic acid to SA. Their data support our hypothesis. Tobacco mosaic virus-inoculated tobacco leaves synthesize SA from the pathway via phenylalanine (YALPANI & al. 1993, RIBNICKY & al. 1998, PALLAS & al. 1996). Therefore, SA seems to be synthesized through a common biosynthesis pathway in ozone-exposed and virus-infected tobacco.

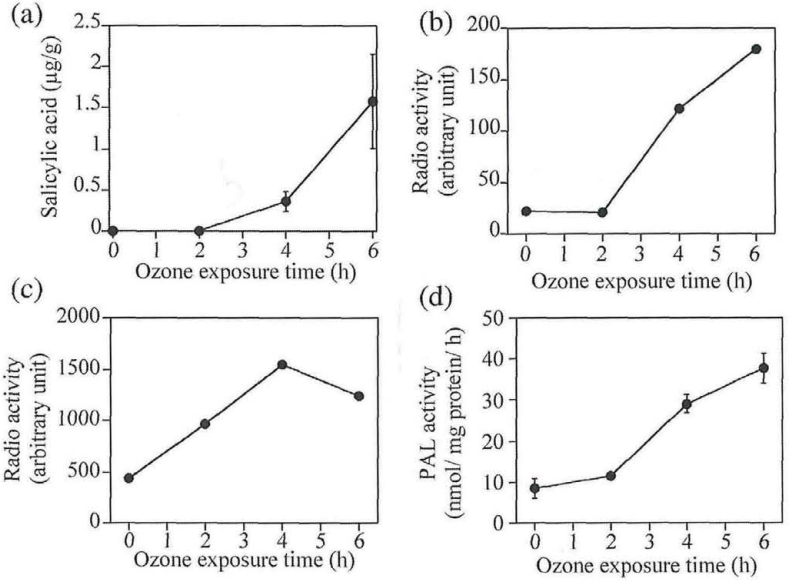

Fig. 2. Ozone induced SA synthesis in tobacco. Changes in (a) SA level, (b) level of *CM* mRNA, (c) level of *PAL A* mRNA and (d) PAL activity. Vertical bars in (a) and (d) represent standard deviations obtained from three replicates.

Because the mRNA level of *ICS1* increased in pathogen-infected *Arabidopsis* leaves (WILDERMUTH & al. 2001), SA may also be synthesized from isochorismate during ozone exposure. To clarify which pathway is used in ozone-exposed *Arabidopsis*, we examined SA level in the *sid2* mutant, in which *ICS1* is defective.

In *Arabidopsis*, SA began to accumulate at 3 h after the start of ozone exposure, and the level kept increasing to 6 h (Fig. 3a). However, SA was not accumulated in *sid2*. In wild-type *Arabidopsis*, the level of *ICS1* mRNA drastically increased after the start of ozone exposure (Fig. 3b). Furthermore, ICS activity at 0 h and 6 h after the treatment was 0 and 0.11 nmol/mg protein/h (± 0.011), respectively, indicating that ICS activity increased in ozone-exposed *Arabidopsis*. These results suggest that SA is synthesized via isochorismate in ozone-exposed *Arabidopsis*. It is reported that SA is synthesized from isochorismate in *Arabidopsis* infected with the fungal biotroph *Erysiphe orontii* and the bacterial necrotroph *Pseudomonas syringae* pv. *maculicola* (WILDERMUTH & al. 2001). This report and our results imply that the SA synthesis pathway partially overlaps between ozone-exposed and pathogen–infected *Arabidopsis*.

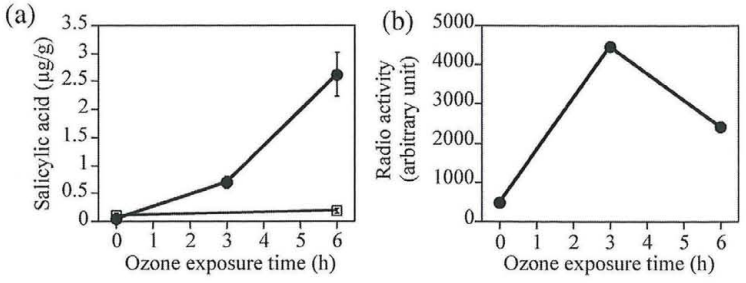

Fig. 3. Ozone induced SA synthesis in *Arabidopsis*. Changes in (a) SA level, (b) level of *ICS1* mRNA. Vertical bars in (a) represent standard deviations obtained from three replicates. ●: wild-type, □: *sid2*.

Thus, our results may suggest that the main pathway of ozone-induced SA biosynthesis differs in tobacco and *Arabidopsis*.

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