Studies on The Pathway and Regulation of Salicylic Acid Biosynthesis in Ozone-Exposed Plants

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Abbreviations

ABA: abscisic acid

ACC: 1-aminocyclopropane-1-carboxylate

ACS: 1-aminocyclopropane-1-carboxylate synthase

AOS: allen oxide synthase

BA: benzoic acid

BA2H: benzoic acid 2-hydroxylase

cDNA: complementary DNA

CM: chorismate mutase

CsCl: Cesium chloride

CTAB: cetyltrimethylammonium bromide

cv.: cultivar

DAB: diaminobenzidine

dCTP: deoxycytidine 5’-triphosphate

dNTP: 2-deoxyribonucleoside triphosphate

DNA: deoxyribonucleic acid

DTT: dithiothreitol

DW: distilled water

EDTA: ethylenediaminetetraacetic acid

EtBr: ethidium bromide

EtOH: ethanol

HPLC: high-performance liquid chromatography

HR: hypersensitive reaction

ICS: isochorismate synthase

IPTG: isopropylthio- β -D-galactoside

JA: jasmonic acid

kb: kilobase pair

KOH: potassium hydroxide

LB: Luria-Bartani

LiCl: lithium chloride

LOX: lipoxygenase

MAPK: mitogen-activated protein kinase

MOPS: 3-[N-morpholino]propanesulfonic acid

mRNA: messenger RNA

NADPH: nicotinamide adenine dinucleotide phosphate

NBT: nitroblue tetrazolium

NaOAC: sodium acetate

OPDA: (9S, 13S)-12-oxophytodienoic acid

PAL: phenylalanin ammonia-lyase

PCD: programmed cell death

PCR: polymerase chain reaction

PEG: polyethylene glycol

PMSF: phenylmethyl-sulfonyl fluoride

PPFD: photosynthetic photon flux density

ppm: parts per million

PR: pathogenesis-related

PVPP: polyvinylpolypyrrolidone

RNA: ribonucleic acid

RNase: ribonuclease

ROS: reactive oxygen species

RT-PCR: reverse transcription-PCR

SA: salicylic acid

SAM: S-adenosylmethionine

SDS: sodium dodecyl sulfate

SSC: standard saline citrate

TBE: tris-borate/EDTA

TCA: trichloroacetic acid

TE: Tris/EDTA solution

TMV: tobacco mosaic virus

Tris: tris(hydroxymethyl)aminomethane

1-MCP: 1-methylcyclopropene

×*g*: gravity

Abstract

Ozone (O_3), a major photochemical oxidant, induces leaf injury concomitant with salicylic acid (SA) synthesis. It is reported that SA has the great effect on determination of O_3 sensitivity in plants. However, SA biosynthesis under O_3 fumigation is not well understood. In plants SA is synthesized via two pathways, involving phenylalanine or isochorismate. When I applied ^{14}C-labeled benzoic acid (BA; a precursor of SA in the pathway via phenylalanine) to O_3-exposed tobacco leaves, it was effectively metabolized to SA. On the other hand, the activity of isochorismate synthase (ICS) and the transcript level of *ICS* were not increased. These results suggest that SA is synthesized via BA from phenylalanine in O_3-exposed tobacco leaves.

Ethylene is a plant hormone that promotes leaf damage in O_3-exposed plants. During O_3 exposure, transgenic plants with a phenotype of reduced O_3-induced ethylene production accumulated less SA than wild-type plants. Ozone increased the activity of phenylalanine ammonia-lyase (PAL) and the transcript levels of the *chorismate mutase* (*CM*) and *PAL* in wild-type tobacco, but their induction was suppressed in the transgenic plants. These results indicate that ethylene promotes SA accumulation by regulating the expression of the *CM* and *PAL* genes in O_3-exposed tobacco.

Increase of SA level, ICS activity and *ICS1* expression level was remarkably

observed in O_3-exposed *Arabidopsis*. However, *ics1* did not show the induction of ICS activity and SA biosynthesis during O_3 exposure, indicating that SA was synthesized from isochorismate in *Arabidopsis*. It is not clarified how SA synthesis in O_3-exposed *Arabidopsis* is regulated. Enhanced *ICS1* expression level and ICS activity were observed during O_3 exposure in SA signaling-deficient plants, *npr1* and NahG. Furthermore, treatment with SA inhibited the increase of *ICS1* expression level during O_3 exposure, suggesting that SA synthesis was negatively controlled by SA signaling.

Taken together, I postulate that these different systems for the regulation of SA synthesis is suitable for the responses induced by SA in tobacco and *Arabidopsis* during O_3 exposure.

Genetral Introduction

Plants cannot move around from their germination site. Accordingly, they have developed the systems to protect from environmental stresses such as low temperature, drought, ultraviolet, air pollution and pathogen. Plants perceive the environmental stress and they respond to avoid or tolerate. For example, in the drought condition plants induce the closure of stomata to inhibit transpiration. Also, plants recognize pathogens and induce cell death for containment of their proliferation. Therefore, understanding the mechanism by which plants perceive environmental signals and transmit to cellular machinery to activate adaptive responses is of fundamental importance to biology.

When plants are exposed to environmental stress, the factors involving signal transduction are induced or activated. Reactive oxygen species (ROS) including O_2^-, H_2O_2 and OH^- are important factors and are produced in 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 ROS during these stresses results from photorespiration, photosynthetic apparatus and mitochondrial respiration. In addition, pathogens, wounding, drought and osmotic stress have been shown to trigger the active production of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes O_2 into O_2^- on the cell membrane

(Hammond-Kosack and Jones 1996; Orozco-Cardenas and Ryan 1999; Cazale et al. 1999; Pei et al. 2000). Calcium ion is a second messenger, and cold, drought, salinity and O_3 have been shown to induce transient Ca^{2+} influx into the cell cytoplasm (Sanders et al. 1999; Knight 2000; Evans et al. 2005). Protein kinase cascade is a conserved signal transduction system in all eukaryotes, and AtMPK6, mitogen-activated protein kinase (MAPK) in *Arabidopsis* is activated by low temperature, pathogen and O_3 (Yuasa et al. 2001, Samuel et al. 2000; Ahlfors et al. 2004). Plant hormones, that are auxin, cytokinin, gibberellin, abscisic acid (ABA), ethylene, brassinosteroid, jasmonic acid (JA) and salicylic acid (SA), are low molecular compounds for regulation of growth and development. The synthesis of ethylene and JA is induced in wound response (Creelman et al. 1992), and SA accumulates in response to pathogen and O_3 (Malamy et al. 1990; Yalpani et al. 1994; Sharma et al. 1996). Thus, plants have multiple signal transduction pathways to respond to environmental stresses.

Defect in these signaling pathways leads to increase of susceptibility to the environmental stress. Loss of AtrbohD and AtrbohF, which are NADPH oxidase, leads to impair ABA-induced stomatal closing, promotion of ROS production, cytosolic Ca^{2+} increase and activation of plasma membrane Ca^{2+}-permeable channels in guard cells

(Kwak et al. 2003). In addition, ABA-deficient mutants, *aba1*, *aba2*, *aba3* displayed a reduced ability to close their stomata (Merlot et al. 2001). From these reports, it is clarified that stomatal closure is required for these signal transduction factors, ABA, ROS and Ca^{2+}. In response to tobacco mosaic virus (TMV), SIPK and WIPK, which are MAPK, are activated in tobacco (Zhang and Klessig 1998). Suppression of SIPK and WIPK attenuates the resistance to TMV, suggesting that these kinases are important to transmit the signal of virus infection (Jin et al. 2003). Thus, the signaling pathways are important for plant in the response to the environmental stresses.

Increase of toxic gaseous pollutants to the tropospheric environment has come from industrialization and urbanization since the 20th centuries. The toxic gases include O_3, sulfur oxide, nitrogen oxide, peroxyacetyl nitrate and fluoride (Nouchi 2002). Ozone is one of the most serious air pollutants, because O_3 is a highly reactive molecule and the environmental concentration of O_3 is increasing. In plants, O_3 penetrates through the stomata, causes leaf damage and then brings about decrease in the productivity of crops and forests (Preston and Tingey 1988). Plant responses to O_3 exposure can be chronic or acute. Visible foliar injury symptoms such as chlorosis, premature senescence and abscission may occur in response to chronic O_3 (Pell et al.

1997). In contrast, acute responses are observed as severe visible leaf injury, when the rates of O_3 uptake exceed the level of detoxification by the plant. Symptoms of acute injury on broad-leafed plants include: chlorosis, bleaching, bronzing, flecking, stippling and necrosis (Kley et al. 1999).

The production of ROS which is induced by acute O_3 exposure was demonstrated by the experiment of nitroblue tetrazolium (NBT) and diaminobenzidine (DAB) staining, which detects O_2^- and H_2O_2, respectively (Rao and Davis 1999; Wohlgemuth et al. 2002). As mentioned above, generation of ROS has shown to be commonly involved in the reaction to various environmental stresses. Thereby, clarifying the mechanism in response to O_3 in plants may lead to elucidate universal reaction in stress response.

It is reported that plant hormones, that are ethylene, JA and SA, play a crucial role to determine the O_3 sensitivity (Kangasjärvi et al. 1994; Rao et al. 2000; Rao and Davis 2001; Overmyer et al. 2003). Ethylene has also been implicated in developmental processes such as the formation of the apical hook in dark-grown seedlings, the 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 (SAM) and

1-aminocyclopropane-1-carboxylate (ACC), and ACC synthase (ACS) is a rate-limiting enzyme for its synthesis. In tomato the expression of *ACSs* (*LeACS1A*, *LeACS2* and *LeACS6*) is induced during O_3 exposure prior to ethylene production (Tuomainen et al. 1997; Nakajima et al. 2001). In O_3-exposed *Arabidopsis* transcript level of *AtACS6* increased (Vahala et al. 1998; Overmyer et al. 2000).

Involvement of ethylene to the formation of O_3-induced leaf injury has been studied from 1970's. At the first time Craker (1971) showed that ethylene production appeared to be related to the extent of injury by comparing two tomato plants with different susceptibility to O_3. Exposure of a range of plant species and cultivars to varying O_3 concentrations demonstrated that the rate of ethylene production correlated with the extent of visible injury (Tingey et al. 1976). When activity of ACS was inhibited by aminoethoxyvinylglycine or suppression of *ACS* expression level, O_3-induced ethylene synthesis decreased and leaf damage was moderated (Mehlhorn and Wellburn 1987; Mehlhorn et al. 1991; Wenzel et al. 1995; Nakajima et al. 2002). Furthermore, it is reported that the propagation of leaf injury in O_3-exposed plant is attenuated by treatment with norbornadiene or 1-methylcyclopropene (1-MCP) that is an inhibitor of ethylene signaling (Bae et al. 1996; Tamaoki et al. 2003). Thus, there are

many reports that ethylene promotes leaf damage in O_3-exposed plants.

Jasmonic acid (JA) inhibits root elongation (Staswick et al. 1992) and is required for pollen development, anther dehiscence (Feys et al. 1994; McConn and Browse 1996; Sanders et al. 2000; Stintzi and Browse 2000), defense against insects (McConn et al. 1997) and necrotrophic pathogens (Thomma et al. 1999). Jasmonic acid is synthesized via a series of steps starting from linoleic acid. The conversion of linoleic acid to (13S)-hydroperoxylinoleic acid by the action of lipoxygenase (LOX), followed by sequential action of allen oxide synthase (AOS) and allen oxide cyclase giving rise to (9S, 13S)-12-oxophytodienoic acid (OPDA), which is JA precursor (Agrawal et al. 2004). It is reported that the expression of *LOX* in soybean and lentil is activated by the treatment with O_3, and that the expression level of *AOS* in O_3-exposed *Arabidopsis* increased (Maccarrone et al. 1992; Maccarrone et al. 1997; Rao et al. 2000). The increase of JA level during O_3 exposure is observed in poplar and *Arabidopsis* (Koch et al. 2000; Rao et al. 2000; Kanna et al. 2003). Exogenous application of JA before O_3 exposure reduces O_3-induced leaf damage (Örvar et al. 1997; Rao et al. 2000; Kanna et al. 2003). In addition, *jar1* with moderated methyl jasmonate inhibition of root elongation displays a greater sensitivity to O_3 (Staswick et al. 1992; Rao et al. 2000;

Overmyer et al. 2000). Therefore, it has been postulated that JA plays a role in reducing damage by O_3 exposure.

Salicylic acid is a key endogenous molecule that mediates disease resistance in many plants (Malamy et al. 1990; Gaffney et al. 1993; Cao et al. 1997), flowering of *Lemna gibba* (Cleland and Ajami 1974) and thermogenicity of *Arum* lilies (Raskin et al. 1987; Raskin 1992). For the induction of disease resistance, SA induced the expression of pathogenesis-related (PR) genes, such as *PR-1*, *ß-1,3 glucanase* and *chitinase* (Carr et al. 1985; Metraux, et al. 1989; Linthorst et al. 1990). Because O_3 activates the expression of *ß-1,3 glucanase* and *chitinase*, involvement of SA in stress response to O_3 had been predicted (Ernst et al. 1992; Schraudner et al. 1992). After these reports Yalpani et al. showed that SA accumulated in O_3-exposed tobacco leaves (1994). The role of SA in O_3-exposed plants is implied from the analysis of transgenic plant (NahG) with an enzyme which catalyzes SA into catechol. This transgenic plant could not accumulate SA in the leaves. Although visible leaf injury is not observed in O_3-exposed *Arabidopsis*, leaf injury is formed in NahG during O_3 exposure (Rao and Davis 1999). This result suggests that SA is required for the induction of defense responses in O_3-exposed *Arabidopsis*. On the other hand, NahG transgenic tobacco showed reduced

leaf injury after O_3 exposure (Örvar et al. 1997), indicating that SA enhances O_3-induced leaf damage in tobacco. Thus, SA appears to have the great effect on determination of O_3 sensitivity in plants but physiological role of SA under O_3 stress condition seems to be confusing. Moreover the pathway of SA biosynthesis and its regulation in O_3-exposed plants is not well understood. It had been thought that SA is synthesized only from chorismate via prephenate, arogenate, phenylalanine, *t*-cinnamic acid and BA in tobacco, rice, cucumber, potato, and *Arabidopsis* (Leon et al. 1993; Yalpani et al. 1993; Silverman et al. 1995; Meuwly et al. 1995; Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998; Ribnicky et al. 1998). Recently, another pathway from chorismate via isochorismate was found in *Arabidopsis* (Wildermuth et al. 2001). Accordingly, two pathways are postulated for SA biosynthesis in plant.

In this thesis, I studied on SA biosynthesis and its regulation in O_3-exposed tobacco and *Arabidopsis*. In Part I, I showed that SA is mainly synthesized from the pathway via phenylalanine in O_3-exposed tobacco and the SA synthesis is enhanced by ethylene action. And in Part II, I indicated that SA is essentially synthesized from isochorismate in O_3-exposed *Arabidopsis* and the SA synthesis is negatively regulated in feedback manner by SA signaling. My research is the first report to clarify the

difference in the regulation of SA synthesis in O_3-exposed tobacco and *Arabidopsis*.

Part I. SA is synthesized from phenylalanine in tobacco and the level of SA under O_3 exposure is regulated by ethylene

1.1 Introduction

It had been thought that SA was synthesized only from phenylalanine via *t*-cinnamic acid and BA in tobacco, potato, and *Arabidopsis* (Leon et al. 1993; Yalpani et al. 1993; Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998; Ribnicky et al. 1998). Phenylalanine ammonia-lyase (PAL) forms *t*-cinnamic acid from phenylalanine (Bate et al. 1994; Howles et al. 1996). The level of SA is diminished in response to TMV inoculation in transgenic tobacco plants in which endogenous PAL expression is suppressed (Pallas et al. 1996). Application of 2-aminoindan-2-phosphonic acid, which is an inhibitor of PAL, inhibits SA accumulation in pathogen-infected *Arabidopsis* and elicitor-treated potato (Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998). These reports suggest that PAL is an important enzyme in the pathway of SA synthesis. In addition, benzoic acid 2-hydroxylase (BA2H) catalyzes the synthesis of SA from BA, and this activity increased in TMV-inoculated tobacco (Leon et al. 1993; Leon et al. 1995), implying that BA2H is also involved in the SA synthesis. On the other hand, Wildermuth et al. (2001) found a new SA synthesis pathway that runs from chorismate via isochorismate in pathogen-infected *Arabidopsis*. It has been reported that *Pseudomonas aeruginosa* has this pathway and that isochorismate synthase (ICS) is the rate-limiting enzyme for SA synthesis in this bacterium (Gaille et al. 2003). Thus,

although these two SA synthesis pathways have been clarified (Fig. 1), the biosynthetic pathway of SA in O_3-stressed plants has not been elucidated. To identify the O_3-inducible SA synthetic pathway in tobacco, I investigated the transcript levels of enzymes involved in SA synthesis.

In the response to O_3, there is a correlation between the rate of ethylene production and the amount of leaf damage (Tingey et al. 1976). Inhibitors of ethylene biosynthesis attenuate injury in O_3-exposed plants (Mehlhorn and Wellburn 1987; Mehlhorn et al. 1991; Wenzel et al. 1995). The introduction of antisense DNA for O_3-inducible ACS can improve O_3 tolerance in tobacco (Nakajima et al. 2002). These results show that ethylene promotes leaf injury after O_3 exposure. It is reported that SA also enhances O_3-induced leaf damage in tobacco (Örvar et al. 1997). Accordingly, ethylene and SA appear to have a common role that regulates the extent of leaf injury after O_3 exposure. However, the details of their roles in O_3-induced damage are unclear. In O_3-exposed tobacco, SA synthesis occurred after induction of ethylene production. Therefore, ethylene seems to regulate SA biosynthesis. In this chapter, I elucidated the effect of ethylene on SA synthesis in O_3-exposed tobacco.

1.2 Materials and Methods

1.2-1 Plant materials and O_3 treatment

Seeds of tobacco (*Nicotiana tabacum* L. cv. SR-1, Bel W3 and 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 day / 20°C night with a relative humidity of 70% and a 14-h light / 10-h dark cycle. Plants were watered daily. I named the fully developed leaf in the highest position the first leaf (see Fig. 7a).

I exposed 4- to 5-week-old plants for 6 h in a chamber to a single dose of 200 $nL \cdot L^{-1}$ O_3 produced by an O_3 generator (Sumitomo Seika Chemicals, Osaka, Japan). Ozone fumigation occurred at 25°C at a relative humidity of 70% under photosynthetic photon flux density (PPFD) of 200 $\mu mol\ m^{-2}\ s^{-1}$ in continuous light. Plants remaining in charcoal-filtered air served as controls.

1.2-2 Extent of leaf injury and ion leakage measurement

The extent of injury was measured as described by Nakajima et al. (2002). Plants were exposed to O_3 for 6 h, then transferred to a fresh-air chamber in continuous light. At 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).

Ion leakage was measured as reported by Tamaoki et al. (2003), with minor modifications. Two leaf disks (diameter, 10 mm) from the second and third leaves were floated on 1 mL of distilled water (DW) for 1 h with shaking at 100 rpm. The conductivity of 100 µL solution from the water bath was determined with a conductivity meter (B-173; Horiba, Kyoto, Japan). The leaves were then autoclaved together with the remaining water (900 µL), and the conductivity of the autoclaved solution was also measured. The ion leakage was obtained by the ratio of conductivity of the pre-autoclaved and autoclaved solution.

1.2-3 SA extraction and quantification

Salicylic acid was extracted from 0.5 g of tobacco leaves and 0.2 g of *Arabidopsis* leaves. Each sample was extracted four times with 1.5 mL methanol. I added 5 µg of *m*-hydroxybenzoic acid as an internal standard. The solution was evaporated to dryness; the residue was dissolved in 100 µL methanol, then 600 µL of 1 mM KOH was added. Lipophilic substances were removed by extraction twice with chloroform. I added 10

μL of phosphoric acid and 700 μL of 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, and the residue was dissolved in 50% methanol and analyzed by HPLC (System Gold, Beckman, CA, USA). Salicylic acid was detected with a fluorescent detector (RF-530, Shimadzu, Osaka, Japan) using E_x = 295 nm and E_m = 370 nm. The mobile phase was 20 mM sodium acetate (pH 2.5) containing 20% methanol.

1.2-4 Preparation of cDNA probes

Complementary DNAs of *CM* and *ICS* in tobacco were isolated by reverse transcription (RT) -PCR using total RNA obtained from O_3-exposed tobacco. The primers for RT-PCR were designed according to the published cDNA sequences (for *CM*, 5′- CTTCAATCTAAGGTTGGTAGAT-3′ and 5′- TTAGTCAAAGGCATAACCCATTC -3′; *ICS*, 5′- ATGCATATCAGTTCTGTTTGCAA -3′ and 5′- CCAGCATACATTCTTCGGTCAAA -3′). The amplified cDNAs were subcloned into a pGEM-T Easy system (Promega, WI, USA) and sequenced with an ALFred sequencer (Amersham Biosciences, NJ, USA).

1.2-5 Expression analysis

Total RNA from leaves was extracted by using an SDS-phenol method. Plant materials were ground using a mortar and pestle with liquid nitrogen. Then 0.5 g of material was transferred to another mortar in which 2 mL of extraction buffer (0.1 M Tris-HCl [pH 8], 50 mM EDTA, and 1% SDS) was added. The solution was transferred to a new tube and lipophilic substances were removed by extraction twice with 4 mL of phenol:chloroform (1:1). The aqueous phase was transferred to a new tube and one third of 10 M LiCl was added. After the incubation at 4°C for 30 min, the solution was centrifuged at 20,000 $\times g$ for 30 min at 4°C. The precipitate was dissolved in 400 µL of TE. The removal of lipophilic substances was carried out by extraction twice with 400 µL of phenol:chloroform (1:1). The aqueous solution was added 40 µL of 3 M NaOAc (pH 5.2) and 1 mL of ethanol, mixed and centrifuged at 17,000 $\times g$ for 10 min at 4°C. The pellet was rinsed by 70% ethanol. After drying under reduced pressure, the pellet was dissolved in 200 µL of DW.

For RNA gel blot analysis, total RNA was separated by electrophoresis through a 1.2% agarose gel that contained 1.8% formaldehyde and then was transferred to a nylon membrane (Hybond N^+; Amersham Biosciences). The probe was prepared by using the

MultiPrime labeling system (Amersham Biosciences) with ^{32}P-dCTP (11 TBq mmol^{-1}). Hybridization was performed at 65°C in a solution containing 0.5 M Na_2HPO_4, 1 mM EDTA, and 7% SDS. Filters were washed in 2×standard saline citrate (SSC), 0.1% SDS at room temperature, then in 0.2×SSC, 0.1% SDS at 58°C. The filter was exposed to a Bio-Imaging Plate (Fuji Film Co., Kanagawa, Japan), and signals were assessed using a Bio-imaging analyzer (BAS2000; Fuji Film Co.).

For RT-PCR total RNA (0.1 μg) was analyzed by PCR with Ready-To-Go RT-PCR Beads (Amersham Biosciences). The primer set used for *PAL A* was 5′-GCACAAAATGGTCACCAAGAAA-3′ and 5′-AAGCCATTGGGGCGACGTTCTA-3′, and that for *PAL B* was 5′-CATGTTAAT-GGAGGAGAAAACT-3′ and 5′-AAGCCATT GTGGAGATGTTCGG-3′.

Reverse transcription reaction was performed at 42°C for 30 min. Polymerase chain reaction was carried out for 15 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 3 min. After electrophoresis in 1.2% agarose gel, the amplified fragments were blotted onto a nylon membrane, hybridized to the cDNA probe, and washed as described above. The nucleotide sequences for cDNAs amplified by the *PAL A* and *PAL B* primers were identical to *PAL A* (AB008199) and

PAL B (AB008120), respectively.

1.2-6 Incorporation of radio-labeled BA into SA

The ^{14}C-labeled BA (about 400 pmol, 2.2 mCi) (American Radiolabeled Chemicals, Inc., MO, USA) was applied to tobacco leaves 4 h after the start of O_3 exposure. Radio-labeled BA was infiltrated from the abaxial surface of the leaf into the apoplast with a 1-mL syringe. Two hours after application, the metabolites of BA were extracted with methanol and the extract was evaporated to dryness. The dried materials were dissolved in 20 mM sodium acetate (pH 5) containing 20% methanol and then analyzed by HPLC. Radioactivity was detected using a liquid scintillation analyzer (2500TR, Packard).

1.2-7 Southern blot analysis

Genomic DNA was extracted from tobacco leaves by cetyltrimethylammonium bromide (CTAB) method. Plant materials were ground using a mortar and pestle with liquid nitrogen. Then 0.2 g of material was transferred to another mortar in which 300 µL of 2% CTAB solution [0.1 M Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl and 2%

CTAB] was added. The solution was incubated at 65°C for 30 min. The solution was added 400 µL of chloroform, mixed and centrifuged at 13,000 $\times g$ for 15 min at room temperature. Lipophilic substances were removed by extraction twice with chloroform. The aqueous phase was transferred to a new tube and 400 µL of 1% CTAB solution [50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% CTAB] was added. After the incubation at room temperature for 30 min, the solution was centrifuged at 9,000 $\times g$ for 5 min. The pellet was dissolved in 400 µL of 1 M CsCl. The solution was added 800 µL of ethanol, incubated at –20°C for 20 min and centrifuged at 13,000 $\times g$ for 5 min at 4°C. The precipitate was rinsed by 70% ethanol. After drying under reduced pressure, the precipitate was dissolved in 100 µL of TE.

For further DNA purification, DNA solution was added 2.3 mL of the solution including 1 M CsCl, 50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.2 mg mL^{-1} EtBr and was transferred to the tube for ultracentrifuge. The solution was added 2.9 mL of 7 M CsCl, 0.1％ Sarcosyl and 0.2 mg mL^{-1} EtBr. The solution was centrifuged at 400,000 $\times g$ for 20 h. The band of linear DNA was collected. The DNA solution was extracted 3 times with equal volume of 2-propanol. The DNA solution was added three volume of TE and 0.6 volume of isopropanol, mixed and centrifuged at 13,000 $\times g$ for 5 min at 4°C. The

pellet was rinsed by 70% ethanol. After drying under reduced pressure, the precipitate was dissolved in 500 μL of TE.

Then, 20 μg of the genomic DNA was digested by restriction enzymes [240 U (μg min^{-1}) of *Dra* I, 200 U of *Eco*R I or 200 U of *Hin*d III] and separated on 1.2% agarose gel. The gel was immersed in 0.25 M HCl for 30 min, then in the second solution including 1.5 M NaCl and 0.5 M NaOH for 45 min, followed in the third solution including 1.5 M NaCl and 0.5 M Tris-HCl, (pH 7.2). The DNA was transferred onto a nylon membrane with 20 × SSC. Preparation of probe, prehybridization and hybridization were performed as described above. The membrane was washed at 42°C with 2×SSC containing 0.1% SDS. Washing was then done at 50°C with 0.2×SSC containing 0.1% SDS.

1.2-8 PAL activity

Enzyme activity for PAL was measured as reported by Legrand et al. (1976), with minor modifications. Plant materials were ground with a mortar and pestle in liquid nitrogen. One hundred μg of the sample was transferred to another mortar with 1 mL of extraction buffer [0.1 M borate buffer (pH 8.8), and 5 mM 2-mercaptoethanol]. After

stirring, the homogenate was centrifuged at 20,000 $\times g$ for 10 min. The supernatant was desalted in a Sephadex G-25 column (NAP-10 Columns, Amersham Biosciences) equilibrated with the extraction buffer. Elute was used as enzyme solution. The extraction was performed at 4°C.

The incubation mixture contained 500 µL of enzyme solution and 33 µL of 2 mM phenylalanine, including L-[U-^{14}C] phenylalanine (28 MBq mol^{-1}). After 1 h of incubation at 37°C, the reaction was stopped by the addition of 33 µL of 18 M sulfuric acid. Five hundred µL of toluene was added to the incubation mixture, and *t*-cinnamic acid was extracted to the organic phase. The radioactivity in the organic phase was determined using a liquid scintillation analyzer (2500TR, Packard, Texas, USA). Protein contents were determined by using a BCA protein assay kit (Pierce, IL, USA).

1.2-9 ICS activity

Extraction and assay of ICS were performed according to the method of Poulsen et al. (1991). Plant materials were ground using a mortar and pestle with liquid nitrogen. Then 1.5 g of material was transferred to another mortar in 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] was added. After stirring, the homogenate was centrifuged at 10,000 $\times g$ for 30 min. The supernatant was desalted in a Sephadex G-25 column (PD-10 Empty Columns, Amersham Biosciences) equilibrated with 0.1 M Tris-HCl (pH 7.5) containing 10 % glycerol, 1 mM EDTA, and 1 mM DTT. Elute was used as enzyme solution. The process was performed at 4°C.

The reaction mixture (total volume 0.5 mL) contained 250 µL of 0.1 M Tris-HCl (pH 7.5), 3 mM Ba-chorismate (Sigma, MO, USA), 15 mM $MgCl_2$, and 250 µL enzyme solution. The reaction was performed for 1 h at 30°C and was stopped by the addition of 125 µL methanol: 2-buthanol (1:1). After centrifugation at 10,000 $\times g$ for 1 min, supernatants were analyzed by HPLC (System Gold, Beckman, CA, USA). Isochorismate was detected with a fluorescent detector (RF-530, Shimadzu, Osaka, Japan) using E_x = 295 nm and E_m = 370 nm. The mobile phase was 20 mM sodium acetate (pH 2.5) containing 20% methanol. The isochorismate produced was quantified by the method of Young and Gibson (1969). A hundred µL of the assay solution after the reaction was added 1.4 mL of 0.1 M phosphate buffer (pH 7.0), and heated at 100 °C for 10 min. By this experiment, isochorismate decomposes to give approximately 25% SA. Salicylic acid was detected by using HPLC.

1.2-10 Establishment of transgenic tobacco As-line4

I constructed an extensively O_3-resistant transgenic tobacco line by the introduction of antisense DNA for *LE-ACS6* isolated from tomato leaves, as reported by Nakajima et al. (2002). The transgenic plants had the same phenotype as that of the AsACS1 previously reported (Nakajima et al. 2002). I named the transgenic tobacco As-line4.

1.2-11 Measurement of ethylene and ACC

Ethylene production was determined as reported by Bae et al. (1996), with minor modifications. At sampling time, the first to third 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 by using a gas chromatograph equipped with a flame ionization detector (GC-7 A; Shimadzu, Osaka, Japan).

I extracted ACC from tobacco leaves and quantified as described by Langebartels et al. (1991). The amount of ACC was determined according to Lizada and Yang (1979). Leaf samples (0.5 g) were extracted 4 times with 1 mL absolute methanol. The extract

was concentrated to dryness and dissolved in 1 mL of water, and lipophilic substances were removed by extraction with chloroform. The upper phase was concentrated to dryness and dissolved in 0.6 mL water. The solution and 0.3 mL of 10 mM $HgCl_2$ was transferred into closed vessel, and 100 μL of saturated NaOH: 10% NaOCl: DW (1:1:1) was added with a syringe. The assay was performed at 4℃ for 1 h. Ethylene produced was quantified with a gas chromatograph equipped with a flame ionization detector.

1.3 Results

1.3-1 O_3-induced leaf injury correlated with SA synthesis

To determine when O_3 treatment induces leaf injury, I measured ion leakage from O_3-exposed tobacco leaves; this value serves as a quantitative indicator of the extent of leaf injury. Ion leakage from the second and third leaves began to increase 4 h after the start of O_3 exposure and reached to 57% and 73% at 8 h, respectively (Fig. 2a). Ion leakage from the first leaf did not increase after O_3 fumigation.

I then examined the accumulation of SA in O_3-exposed tobacco. Salicylic acid started to accumulate at 4 h and was increased more at 6 h (Fig. 2b). In addition, SA accumulation occurred concomitantly with the increase of ion leakage from leaves (Figs. 2a, b). In the absence of O_3, SA was not detected.

1.3-2 The expression of *CM* and *PALs*, but not *ICS*, was induced by O_3 treatment

Two SA synthesis pathways have been reported in pathogen-infected plants: one is regulated by chorismate mutase (CM), PAL and BA2H, and the other is regulated by isochorismate synthase (ICS) and pyruvate lyase (Fig. 1; Shah 2003). However, the pathway used for SA synthesis in O_3-stressed tobacco has not yet been evaluated. To

examine the levels of transcript for these enzymes, I isolated partial cDNAs encoding *CM*, *PAL*, and *ICS* by RT-PCR of total RNA from 4- to 5-week-old tobacco with O_3 exposure. The deduced amino acid sequence of tobacco CM (AB182997) was 79% identical to that of the LeCM of *Lycopersicon esculentum* (Q9STB2) (Fig. 3a). The deduced amino acid sequence of tobacco ICS (AB182580) was compared with another plant ICS and showed high homology to that of CrICS from *Catharanthus roseus* (93% identity) (Fig. 3b). To clarify the number of *ICS* in a tobacco genome, I carried out Southern blot analysis by using tobacco *ICS* as a probe. The nucleotide sequence of *ICS* in tobacco has a restriction site for *Dra* I, but not for *Eco*R I or *Hin*d III. When tobacco total DNA was digested with *Dra* I, I identified two hybridization signals (Fig. 3c). On the other hand, I detected only a single band with *Eco*R I or *Hin*d III digestion. This result suggested that tobacco *ICS* might be a single copy in a genome. From the structural features of tobacco *ICS* and the results of Southern blot analysis, *ICS* in tobacco might encode an ortholog of *CrICS* in *Catharanthus roseus*.

Tobacco has two PAL genes, *PAL A* (AB008199) and *PAL B* (AB008200), and the identity of their DNA sequences is high (85% identity). To distinguish the transcript levels of *PAL A* and *PAL B*, I constructed specific primers for these genes, and then

performed RT-PCR as a tool for expression analysis. The transcript level of *CM* increased slightly after O_3 exposure (Fig. 4a). Transcript levels for *PAL A* and *PAL B* began to increase at 2 h, peaked at 4 h, and had slightly decreased at 6 h (Fig. 4b). Transcript levels of *ICS* in tobacco, however, did not increase with O_3 exposure (Fig. 4a). Moreover, ICS activity in O_3-exposed tobacco was not detected, whereas the level of SA was increased (Fig. 2b). These results suggest that O_3-induced SA synthesis in tobacco leaves might occur through an increase in the transcript levels of *CM*, *PAL A*, and *PAL B*, but not by participation of *ICS*.

To investigate more clearly which pathway is used for SA synthesis in O_3-exposed tobacco, I applied a radio-labeled ^{14}C-BA, a precursor of SA, to O_3-fumigated leaves and measured the incorporation of radioactivity into SA. When ^{14}C-BA was applied, O_3-exposed and control plants metabolized 3.8% (±1.3%) and 0.9% (±0.4%) of radioactivity to SA and respectively. The incorporation of radioactivity into SA in O_3-exposed plants was about four times as high as that in control plants (Fig. 5). This result also indicates that the pathway of SA synthesis via BA is induced when tobacco plants are fumigated with O_3.

1.3-3 The increase of SA level was inhibited in transgenic tobacco with reduced ethylene production during O_3 exposure

Ethylene emission in tobacco began to increase 1 h after O_3 exposure (Fig. 6b), whereas SA accumulation began to increase at 4 h (Fig. 2b). Because SA synthesis delayed from ethylene production, ethylene may regulate SA synthesis. To clarify whether ethylene participates in the regulation of SA synthesis during O_3 exposure, I carried out a physiological analysis of transgenic tobacco (As-line4) expressing anti-sense DNA for O_3-inducible *ACS* (*LE-ACS6*) from tomato. In the As-line4 plants, anti-sense RNA for *LE-ACS6* was constitutively detected (Fig. 6a).

As As-line4 is a new transgenic line and the experimental conditions differed from previous report (Nakajima 2002), I compared the levels of ethylene production in wild-type tobacco and As-line4 during O_3 exposure. In wild-type plants, ethylene emission began to increase 1 h after O_3 exposure and the value at 6 h was the highest (Fig. 6b). By contrast, ethylene production in As-line4 was about half that in wild-type tobacco at 4 and 6 h. Moreover, the level of 1-aminocyclopropane-1-carboxylate (ACC), a precursor of ethylene was increased 6-fold 4 h after the start of O_3 fumigation in wild-type plants, whereas in O_3-exposed As-line4 plants it was half that in the wild-type

(Fig. 6c).

When wild-type plants were exposed to 0.2 ppm O_3 for 6 h, their leaves withered and then turned dark brown 18 h after the exposure. Spotted injuries appeared on the first leaf, and extensive wilting occurred in the second and third leaves (Fig. 7a). In transgenic plants, leaf damage was moderated, and the extent of leaf injury in the first, second, and third leaves of As-line4 was less than that in the wild-type (Fig. 7b).

Ion leakage from the second and third leaves of wild-type plants began to increase at 4 h and reached 55% and 76%, respectively, 8 h after the start of O_3 exposure (Fig. 7c). By contrast, ion leakage from the second and third leaves was 25% and 33%, respectively, in As-line4 plants after 8 h of O_3 exposure. Neither type of plant showed increased ion leakage in the first leaves.

In wild-type plants, SA started to accumulate at 4 h and reached the highest level at 6 h (Fig. 8a). Although SA accumulation in As-line4 plants also reached the highest level at 6 h, the amount was one-fifth that of wild-type tobacco.

1.3-4 Ethylene enhances expression of enzymes to form the precursor of SA

I compared the transcript levels associated with SA synthesis in O_3-exposed wild-type and transgenic plants. In wild-type plants, the transcript levels of *CM*, *PAL A*, and *PAL B* increased remarkably after the onset of O_3 exposure (Fig. 9a). Their transcript levels in O_3-exposed As-line4 also increased, but to lesser extent. The activity of PAL in wild-type plants began to increase 2 h after O_3 exposure and the value at 6 h was the maximum (Fig. 8b). However, PAL activity in As-line4 6 h after the start of O_3 exposure was half that in wild-type plants. It is well known that Bel W3 is sensitive to O_3 and its fraternal cultivar Bel B is tolerant to it (Heggestrad et al. 1991). In fact, Bel W3 showed more sensitive phenotype than Bel B when exposed to 0.2 ppm O_3 for 6 h (Fig. 10a). After O_3 exposure Bel W3 produced a greater amount of ethylene as compared to Bel B (Fig. 10b). To confirm whether ethylene regulates SA level in non-transgenic plants, I examined SA level in O_3-exposed Bel W3 and Bel B. Bel W3 synthesized higher amount of SA than Bel B 4h and 6h after the start of O_3 exposure (Fig. 10c). When I compared the transcript levels of *CM*, *PAL A*, and *PAL B* during O_3 exposure in both plants, Bel W3 accumulated higher transcripts of these genes than Bel

B (Fig. 11a and b). These results indicate that plants producing more O_3-induced ethylene have higher levels of SA by enhanced expression of those enzymes. Therefore, my results suggest that O_3-induced ethylene production enhances the levels of transcript of *CM*, *PAL A* and *PAL B*.

1.4 Discussion

1.4-1 SA-induced leaf injury in O_3-exposed tobacco.

I showed here that O_3 induced SA accumulation and increase of ion leakage in tobacco (Figs. 2a and b). The extent of leaf injury in transgenic tobacco plants (NahG) with ectopic expression of *NahG*, which fail to accumulate SA, is attenuated in comparison with that in wild-type tobacco (Örvar et al. 1997). Their report and my results support the hypothesis that SA leads to the formation of leaf damage in tobacco after O_3 treatment.

Ion leakage in the first leaves did not increase in my study, whereas that in the second and third leaves increased (Figs. 2a and 7c). The reason why younger leaves were more resistant to O_3 might be their higher activity of superoxide dismutase, which converts superoxide radicals into H_2O_2, and/or their increased content of ascorbate, which serves as an antioxidant (Lee and Bennett 1982; Morabito and Guerrier 2000).

1.4-2 Tobacco synthesizes SA from phenylalanine.

I showed that O_3 exposure simultaneously induced increases in the transcript levels of *CM*, *PAL A*, and *PAL B* and in PAL enzyme activity (Figs. 4a, b and 9b). Furthermore, radio-labeled BA was metabolized to SA more effectively in O_3-exposed

tobacco (Fig. 5). These results indicate that O_3 induced SA synthesis by way of phenylalanine and BA. This hypothesis is supported by the results of Pasqualini et al. (2002), who reported that in tobacco O_3 increased the activity of BA2H, which catalyzes BA to SA. On the other hand, no ICS activity was detected when O_3 was fumigated (Fig. 4a). Furthermore, low transcript level of *ICS* was detected and did not increase in both control and O_3-exposed tobacco. These results also fortify that O_3-induced SA synthesis in tobacco occurs through a pathway involving phenylalanine.

1.4-3 Ethylene enhances SA synthesis in O_3-exposed tobacco

It is well known that ethylene and SA promote the formation of leaf injury. In my results, ethylene synthesis was induced before the start of SA accumulation in O_3-exposed tobacco (Figs. 2b and 6b). Furthermore, increases in the levels of SA and PAL activity and transcripts for *CM*, *PAL A*, and *PAL B* were suppressed in As-line4 plants (Figs. 8 and 9). Accordingly, my results suggest that ethylene enhances SA synthesis through up-regulation of the transcript levels of *CM*, *PAL A*, and *PAL B*.

Bel W3 emitted ethylene more than Bel B when exposed to O_3 (Fig. 10b).

Pasqualini et al. (2002) had shown that Bel W3 accumulates higher levels of SA than Bel B after O_3 exposure. In my experiments, SA level and transcripts for *CM*, *PAL A*, and *PAL B* increased to a greater extent in Bel W3 plants than in Bel B plants (Figs. 11a and b). These findings support the hypothesis that the level of SA is controlled by ethylene during O_3 exposure.

It had been reported that ethylene promotes SA signaling and accumulation in plants. For example, *ethylene insensitive 2* (*ein2*) attenuates SA-dependent cell death of *accelerated cell death 5* (*acd5*) in *Arabidopsis* (Greenberg et al. 2000). Moreover, SA does not accumulate in ethylene-deficient and ethylene-signaling-deficient tomato infected with a pathogen (O'Donnell et al. 2001). Although these reports suggested that ethylene induces SA synthesis under O_3-stressed conditions, they had not mentioned how ethylene enhances SA synthesis. My results demonstrate for the first time that ethylene promotes *CM*, *PAL A*, and *PAL B* expression to increase SA synthesis in O_3-exposed tobacco leaves.

Contrary to my report, Rao et al. (2002) found that the NahG-transformed *Arabidopsis* and the *npr1* mutant, which lack SA signaling, failed to produce ethylene in response to O_3; they concluded that SA stimulates ethylene synthesis in O_3-exposed

plant. Why is there the apparent discrepancy? Overmyer et al. (2000) proposed a hypothesis that SA and ethylene promote the synthesis of each other through the generation of ROS. By their hypothesis, the inconsistency between my data and the results of Rao et al. (2002) can be explained. However, in O_3-exposed tobacco ethylene must trigger SA synthesis because ethylene emitted prior to SA accumulation (Figs. 2b, 6b).

I showed that O_3-induced SA in tobacco was synthesized from phenylalanine via *t*-cinnamic acid and BA, and that O_3-induced ethylene promoted SA accumulation. Does *Arabidopsis* synthesize SA from phenylalanine during O_3 exposure? In the next chapter, I address to answer this question.

Part II. SA accumulation under O_3 exposure is controlled by feedback inhibition of SA signaling in O_3-exposed *Arabidopsis*

2.1 Introduction

From the phenotype analysis of NahG transgenic *Arabidopsis*, in O_3-exposed *Arabidopsis* SA seems to play a role to trigger defense reaction, involving the induction of antioxidant enzymes (Rao and Davis 1999). However, despite its importance in plant defense against O_3 exposure, the regulation of SA biosynthesis in ozone-exposed plant had not been revealed.

There are two pathways for SA biosynthesis in plants as described in Introduction of Part I. The accumulation of SA in pathogen-infected *Arabidopsis* was inhibited by application of 2-aminoindan-2-phosphonic acid, an inhibitor of PAL (Mauch-Mani and Slusarenko 1996), suggesting that SA is synthesized from the pathway via phenylalanine. In contrast, decreased SA level was shown in pathogen-infected *salicylic acid induction deficient 2* (*sid2*) mutant, which defects isochorismate synthase 1 (ICS1), indicating that SA synthesis occurs through the pathway via isochorismate in pathogen-infected *Arabidopsis*. Thus, different results were reported. In O_3-exposed tobacco, SA was synthesized from the pathway via phenylalanine as described in Part I. To clarify which pathway SA is synthesized from in O_3-exposed *Arabidopsis*, I investigated the pathway of SA biosynthesis during O_3 exposure.

Elevated SA level and *PR-1* expression was exhibited in *Arabidopsis* mutants,

such as *constitutive expresser of PR genes* (*cpr1*, *cpr5* and *cpr6*), *constitutive immunity* (*cim5-14*, except for *cim8*), and *lesions simulating disease* (*lsd1*) (Bowling et al. 1994; Bowling et al. 1997; Clarke et al. 1998; Maleck et al. 2002). However, it still remains to elucidate how SA level was regulated in O_3-exposed *Arabidopsis*. I have shown that ethylene regulated SA synthesis in O_3-exposed tobacco as described in Part I. Moreover, it is reported that SA synthesis in pathogen-infected plant was negatively regulated by Non expressor of PR genes 1 (NPR1) protein, which is the factor mediating SA signaling (Cao et al. 1997). To reveal how SA level is controlled in O_3-exposed *Arabidopsis*, I studied the implication of ethylene and SA signaling on SA biosynthesis.

2.2 Materials and Methods

2.2-1 Plant materials and O_3 treatment

Seeds of *Arabidopsis thaliana* cv. Col-0 and mutants were sown on blocks of glass wool (2 x 2.3 cm, Nittobo, Tokyo, Japan). Then, they were placed at 4°C for 2 days for vernalization treatment. Seedlings were grown in a growth chamber at 22°C, under the 14 h-light with PPFD of 100 μmol m^{-2} s^{-1} from white fluorescent lamps, at 50-60% relative humidity. Sixteen-day-old seedlings were exposed to 200 nL L^{-1} of O_3 in a growth chamber at 25°C under continuous light with PPFD of 100 μmol m^{-2} s^{-1}. Relative humidity was kept at 70%.

2.2-2 Preparation of cDNA probes

Complementary DNAs of *ICS1* (At1g74710), *ICS2* (At1g18870), *CM1* (At3g29200), *CM2* (At5g10870), *CM3* (At1g69370), *PR-1* (At2g14610) and *PR-2* (At3g57260) in *Arabidopsis* were isolated by RT-PCR using total RNA obtained from O_3-exposed *Arabidopsis*. The primers for RT-PCR were designed according to the published cDNA sequences of *Arabidoopsis* (for *ICS1*, 5′-GTTCCAATTGACCAGCAAATCGG-3′ and 5′-CTGAGGGACTGAAAAGTAAAATG-3; *ICS2*,

5′-GTACCAATTGAGCAGAAAATTGG-3′ and 5′-CTTCGGATTGATCTCCAGTCATC-3′; *CM1*, 5′-TTCTTCCCTGTCAACGATCCAGC-3′ and 5′-TTAGCTTGGATGATTTCTGTCTC-3′; *CM2*, 5′-ATGGCAAGAGTCTTCGAATCGGA-3′ and 5′-ATCTTCTACGTCGTCTCGATTGA-3′; *CM3*, 5′-ATGGAGGCTAAGTTACTCAAACC-3′ and 5′-TTAATCCAGTCTTCTAAGCAAGT-3′). *PR-1*, 5’-CTTTGTAGCTCTTGTAGGTGC-3’and 5’-ACACCTCACTTTGGCACATCC-3’, *PR-2*, 5’-CTTCCTTCTTCAACCACAC-3’ and 5’-CTCATACGTTGGTTCCTTC-3’). The amplified cDNAs were subcloned into a pGEM-T Easy system (Promega, WI, USA) and sequenced with an ALFred sequencer (Amersham Biosciences, NJ, USA). The cDNA fragments of *PAL1* (At2g37040) (ABRC stock no. CD3-121), *PAL2* (At3g53260) (ABRC stock no. CD3-122), and *PAL3* (At5g04230) (ABRC stock no. CD3-123) were obtained from ABRC. These cDNA fragments were sequenced for confirmation before use.

2.2-3 SA treatment

I applied 0.1 mM SA with 0.05% Tween 20 to 16-day-old seedlings by a sprayer. Then, the seedlings were exposed to 200 nL L^{-1} of O_3. As control, 0.05% Tween 20 were sprayed to seedlings.

2.2-4 1-MCP treatment

For treatment with 1-methylcyclopropene (1-MCP), 0.8 μM of 1-MCP was dissolved with 804 μL of DW in a tube, and plants were placed with the tube in a closed chamber (67 L) for 12 h. The final concentration of 1-MCP in the gas phase of the chamber was 500 pL L^{-1}.

2.3 Results

2.3-1 SA was not accumulated in *ics1* by O_3 treatment

To reveal which pathway is used for SA synthesis in O_3-exposed *Arabidopsis*, I examined expression level of *CM*, *PAL* and *ICS*. *Arabidopsis* has three kinds of *CM* (*CM1, CM2, CM3*), three kinds of *PAL* (*PAL1, PAL2, PAL3)* and two kinds of *ICS* (*ICS1* and *ICS2*) as candidate of genes for SA synthesis. I investigated the mRNA level of these genes in O_3-exposed *Arabidopsis* by using RNA blot analysis. Accumulation of transcripts for *CM3*, *PAL1*, *PAL2* and *ICS1* was detected by this analysis whereas mRNA from *CM1*, *CM2*, *PAL3* and *ICS2* was under detectable level (Fig. 12a). Although transcript level of *ICS1* was remarkably induced 3 h after the start of O_3 exposure, the slight increase of transcripts for *PAL2* was observed at 3h. Transcript level of *CM3* and *PAL1* did not increase by O_3 treatment.

To clarify the importance of *ICS1* for SA synthesis, I determined the ICS activity and SA level in *ICS1*-deficient mutant (*ics1*). The activity of ICS in wild-type *Arabidopsis* began to increase 3 h after the start of O_3 exposure, reached highest level at 6 h and then decreased whereas the increase was not observed during O_3 exposure in *ics1* (Fig. 12b). Although the accumulation of SA in wild-type started at 3 h during O_3 exposure and increased until 9 h, SA was not accumulated in *ics1* (Fig. 12c). The

expression of *PR-1*, the SA-inducible gene, was induced in O_3-exposed wild type *Arabidopsis*, whereas it was not induced in O_3-exposed *ics1* (Fig. 12d).

2.3-2 The level of SA in ethylene insensitive mutants during O_3 exposure were equivalent to that of wild-type

The regulation of SA biosynthesis in O_3-exposed plant has not been revealed. Endogenous ethylene enhanced SA synthesis by regulating the genes expression of *CM* and *PAL* in O_3-exposed tobacco as described in Part 1. To determine whether ethylene regulates SA biosynthesis in O_3-exposed *Arabidopsis*, I examined SA level in *etr1* and *ein2*, which defects ethylene signaling. The level of SA during O_3 exposure was similar between wild-type and ethylene insensitive mutants (Fig. 13). Furthermore, I examined SA synthesis in plant treated with 1-MCP, an inhibitor of ethylene reception. The expression level of *PR-4*, a marker for ethylene signaling, in 1-MCP-pretreated plant during O_3 exposure was lower than that in control (Fig. 14a), indicating that ethylene signaling was inhibited by treatment with 1-MCP. However, SA level in 1-MCP-pretreated *Arabidopsis* was same to that in control (Fig. 14b). These results suggest that ethylene might not regulate SA level in O_3-exposed *Arabidopsis*.

2.3-3 Enhanced increase of *ICS1* expression and ICS activity during O_3 exposure were observed in SA signaling-deficient plants

To clarify the effect of SA signaling on SA biosynthesis, I analyzed the ability of SA production in the mutant of *npr1* and the transgenic plant of NahG (NahG). The *npr1* and NahG defected SA inducible *PR-1* expression in response to pathogen (Cao et al. 1997; Lawton et al. 1995), and this phenomenon was also observed during O_3 exposure (Fig. 15a and b). This result indicates that SA signaling pathway is also deficient under O_3-exposed condition in *npr1* and NahG. The SA level of *npr1* during O_3 exposure was 4-fold higher than that of wild-type 12 h after the start of O_3 exposure (Fig. 13), implying that SA level in O_3-exposed *Arabidopsis* is negatively regulated by SA signaling. The accumulation of SA was almost completely suppressed in NahG.

To elucidate how SA level is regulated by SA signaling, I examined *ICS1* expression during O_3 exposure in SA signaling-deficient plants, *npr1* and NahG. The levels of *ICS1* expression between wild-type and *npr1* were similar 3 h after the start of O_3 exposure but the expression level in *npr1* at 6 h and 12 h was higher than that in wild-type (Fig. 15a). The level of *ICS1* expression in NahG during O_3 exposure was

also higher than that in wild-type (Fig. 15b). Furthermore, enzyme activity of ICS in *npr1* was activated to a similar level of wild-type 3 h after the start of O_3 exposure, and the activity in *npr1* was at least 2-fold higher than that in wild-type at 6 h, 9 h and 12 h. The transgenic NahG plant also displayed more enhanced activity of ICS than wild-type at 6 h, 9 h and 12 h (Fig. 15c). These results showed that SA-deficient plants accumulated the greater amount of *ICS1* transcripts and ICS activity during O_3 exposure.

To reveal in detail the role of SA on the ability of SA synthesis during O_3 exposure, I investigated *ICS1* expression and ICS activity in O_3-exposed *Arabidopsis* with or without SA treatment. While *ICS1* expression was induced in O_3-exposed *Arabidopsis* without SA treatment, the induction was suppressed in SA-treated plant (Fig. 16a). Moreover, increase of ICS activity was observed after O_3 exposure whereas the increase was diminished by SA treatment (Fig. 16b). These results suggest that the high amount of SA negatively regulates *ICS1* expression in O_3-exposed *Arabidopsis*.

2.4 Discussion

2.4-1 SA is synthesized from isochorismate in O_3-exposed *Arabidopsis*

In this study, I examined SA synthesis pathway in O_3-exposed *Arabidopsis*. The level of *ICS1* expression drastically increased 3 h after O_3 exposure (Fig. 12a). The remarkable increase of the expressions of the other genes involving SA synthesis was not observed during O_3 exposure. Furthermore, ICS activity was induced by O_3 exposure in *Arabidopsis* (Fig. 12b). The level of SA increased in O_3-exposed wild-type *Arabidopsis*, but not in O_3-exposed *ics1*, which defects ICS activity (Figs. 12c and d). These results suggest that SA is synthesized from isochorismate by induction of *ICS1* gene expression in O_3-exposed *Arabidopsis*. It is reported that SA is synthesized from the pathway via phenylalanine and isochorismate in pathogen-infected *Arabidopsis* (Mauch-Mani and Slusarenko 1996; Wildermuth et al. 2001). Because the increase of SA level was not activated in pathogen-infected *ics1*, it is highly possible that SA is mainly synthesized from isochorismate in response to pathogen infection (Wildermuth et al. 2001). Therefore, O_3-exposed *Arabidopsis* appear to synthesize SA by the same pathway in response to pathogen attack.

2.4-2 SA accumulation under O_3 exposure is controlled by feedback inhibition of SA signaling in *Arabidopsis*

When I checked SA accumulation during O_3 exposure in ethylene insensitive mutants, *etr1* and *ein2*, the level of SA was similar to that in wild-type (Fig. 13). Furthermore, although inhibition of SA signaling in O_3-exposed *Arabidopsis* was observed by pretreatment with 1-MCP, there is no difference in SA accumulation of 1-MCP-pretreated plant and control (Fig. 14b). These results suggest that ethylene was not involved in SA biosynthesis of *Arabidopsis.*

The mutant of *npr1* during O_3 exposure induced higher level of SA, *ICS1* expression and ICS activity rather than wild-type (Figs. 13, 15a and c). Treatment with SA inhibited the increase of *ICS1* expression level and ICS activity by O_3 exposure (Fig. 16a and b), implying that SA synthesis is controlled by negative feedback regulation of SA signaling in *Arabidopsis* and NPR1 negatively regulates SA synthesis. It has reported that the level of *ICS1* mRNA and SA in *npr1* elevated as compared to wild-type when pathogen was infected (Wildermuth et al. 2001). Their data support a role for NPR1 as a negative regulator of *ICS1* expression. Level of *ICS1* expression and ICS activity in O_3-exposed NahG also were higher than that of wild-type (Figs. 15b and

c). Intriguingly, the enzyme activity for ICS in NahG at 9 h and 12 h was significantly higher than that in *npr1* (Fig. 15c). Why this difference comes from? It has been reported that there are two pathways for SA signaling, namely NPR1-dependent pathway and NPR1-independent pathway (Grazegrook 2005). As NahG may completely convert SA to catechol, it lacks both SA signaling pathways. On the other hand, *npr1* defect only NPR1-dependent pathway. Both SA signaling pathways, therefore, might be participated in the negative feedback regulation of *ICS1* expression. Given this idea, the difference of ICS activity between *npr1* and NahG during O_3 exposure may be attributed to the difference of participation to inhibit *ICS1* expression by SA signaling. On the contrary to my results, expression of *ICS1* was not remarkably altered in pathogen-infected NahG transgenic plants (Wildermuth et al. 2001), suggesting that the regulation mechanism of *ICS1* expression may have a difference between pathogen-infected plants and O_3-exposed plants.

The protein of PchA, which is ICS in *Pseudomonas aeruginosa*, limited the production of SA, suggesting that ICS is rate-limiting enzyme in SA biosynthesis (Gaille et al. 2003). The mutant of *ics1* did not induce the activity of ICS and SA accumulation during O_3 exposure (Fig. 12 b and c). In O_3-exposed *Arabidopsis*, the

peak time of *ICS1* expression, the value of ICS activity and SA level is observed 3 h, 6 h and 9 h, respectively, indicating that SA accumulation accompanies increase of *ICS1* expression and ICS activity (Fig. 12a, c and 15c). In addition, *npr1* with higher O_3-induced ICS activity exhibited the higher SA level during O_3 exposure, showing that accumulation of SA is associated with the increase of ICS activity. Therefore, ICS may be the rate-limiting enzyme in *Arabidopsis* as *Pseudomonas aeruginosa*.

In conclusion, this study is the first report to show that SA level is regulated by feedback inhibition from SA signaling in O_3-exposed *Arabidopsis*. It is unknown at present what factors other than NPR1 in SA signaling pathways were associated with suppression of *ICS1* expression. Accordingly, future studies are needed to clarify factors of SA signaling pathway responsible for this negative feedback regulation.

General Discussion

In Part I, I showed that SA is mainly synthesized from phenylalanine in O_3-exposed tobacco and the level of SA under O_3 exposure is regulated by ethylene. In Part II, I indicated that the main pathway of SA biosynthesis is the pathway from isochorismate in O_3-exposed *Arabidopsis* and the SA accumulation during O_3 exposure is controlled by feedback inhibition of SA signaling. In this study, I clarified that the regulatory system of SA biosynthesis is different in both tobacco and *Arabidopsis*. Why SA synthesis is regulated by the different mechanism?

It is reported that the response to O_3 is similar to hypersensitive reaction (HR) (Schraudner et al. 1998; Rao and Davis 2001), which is the phenomenon for containments of the pathogen at the infection site (Greenberg, 1997; Heath, 2000). The production of an excessive amount of ROS and SA accumulation are induced in HR (Baker and Orlandi 1995; Lamb and Dixon 1997; Malamy et al. 1990). The drastic increase of ROS level is observed in Bel W3 after O_3 exposure, but not during O_3 exposure, whereas the increase was not observed in Bel B (Schraudner et al. 1998). In O_3-exposed Bel W3, high level of SA accumulation was induced in this study. Thereby, there is a possibility that the response to O_3 in tobacco mimics HR.

It has been shown that HR includes programmed cell death (PCD). In

multicellular organisms, PCD is a genetically encoded system for suicide of the cells that plays a central role to development and homeostasis (Ameisen 2002). Cytochrome c, an essential component of the respiratory chain for the generation of ATP, functions as a trigger of PCD. Cytochrome c normally resides in the space between the outer and inner membrane of mitochondria. Its release into cytoplasm induced by stress or development cues leads to activation of caspase, which is a kind of cystein protease, and then DNA fragmentation and chromatin condensation occur. Specific inhibitors of animal caspase-1 and -3 could attenuate the induction of HR in bacteria- or TMV-infected tobacco leaves (del Pozo and Lam, 1998). In addition, it is reported that vacuolar processing enzyme with a caspase-1 activity is isolated from tobacco and mediate HR (Hatsugai et al. 2004). From these reports, PCD may be required for the induction of HR. In O_3-exposed tobacco Bel W3, the phenomenon of PCD was observed such as release of cytochrome c from mitochondria, activation of cystein protease, DNA fragmentation and chromatin condensation (Pasqualini et al. 2003). Therefore, PCD might be also induced in O_3-exposed plant. It is possible that the response to O_3 mimics HR not only from the accumulation of ROS and SA but also from the induction of PCD. Because high level of SA was observed in O_3-exposed Bel

W3 in my study, SA may be a key signal for induction of PCD.

Transgenic tobacco of NahG showed reduced leaf injury during O_3 exposure (Örvar et al. 1997), indicating that SA plays a role to enhance O_3-induced leaf damage. Why tobacco induces SA biosynthesis in response to O_3? There is a possible explanation that tobacco prevents from absorbing O_3 by utilizing SA-induced PCD system. Because ethylene enhances SA accumulation in O_3-exposed tobacco, it is presumable that tobacco has the proper system to amplify SA synthesis in response to O_3 for triggering PCD and then to promote the formation of leaf injury.

In contrast, visible leaf injury was not observed in O_3-exposed *Arabidopsis*. In O_3-exposed NahG transgenic *Arabidopsis*, leaf injury was propagated, and induction of antioxidant enzymes including superoxide dismutase was lower level than that in wild-type (Rao and Davis 1999). Their results suggest that SA functions as an inducer of defense responses in O_3-exposed *Arabidopsis*. However, it is reported that SA also has toxic function. Dwarf and cell death phenotypes are observed in constitutively SA accumulation mutants, such as *constitutively activated cell death 1* (*cad1*). Overexpression of *NahG* and the mutation of NPR1 almost completely restored the cell death phenotype in *cad1* (Morita-Yamamuro et al. 2005), demonstrating that

constitutively high level of SA is detrimental for plant. Thus, SA plays a role to induce the defense system to O_3 exposure in *Arabidopsis* whereas high level of SA retards the plant growth and development. Because plants need to decrease SA level to avoid its toxic effects, *Arabidopsis* may have the feedback inhibition system for SA synthesis.

In conclusion, as tobacco and *Arabidopsis* have the different SA role, each appropriate system in response to O_3 has evolved and the regulation of SA synthesis may consequently be different. It is presumable that both reactions must be suitable for plant to respond to O_3 exposure. Taken together, I demonstrated the difference of regulation of SA synthesis in tobacco and *Arabidopsis*. My results are one of the examples showing biodiversity of stress response. Further analysis of the regulation system of SA biosynthesis in various plant species will address the elucidation of common or specific response to O_3 in plants.

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Figures

Figure 1. Proposed pathways of SA biosynthesis.

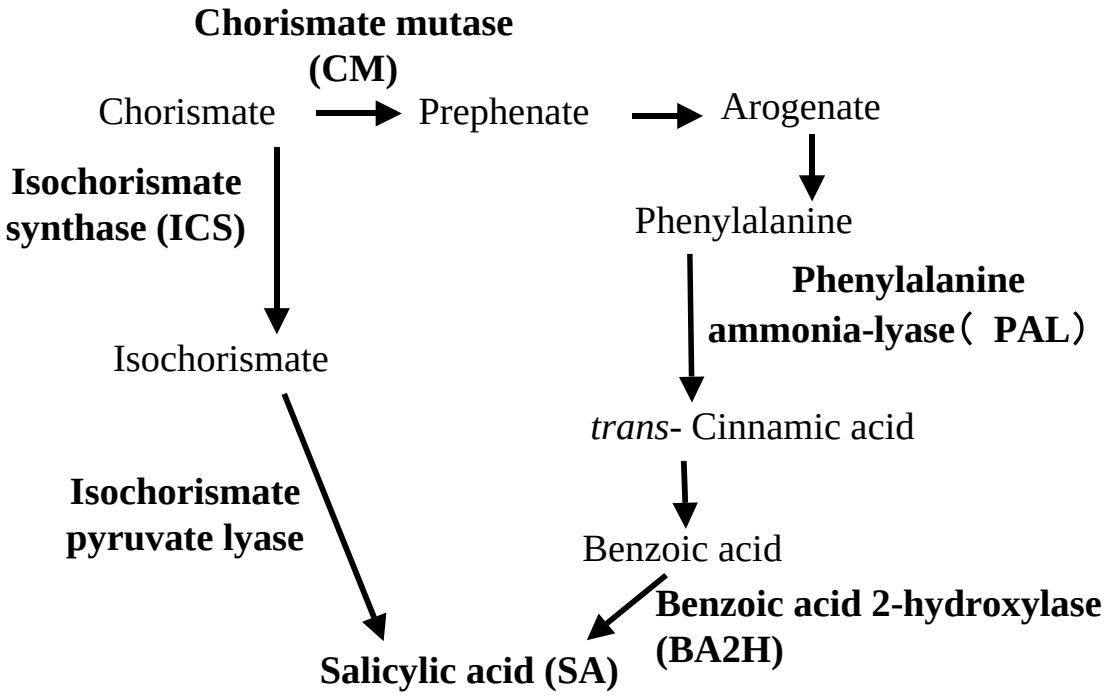
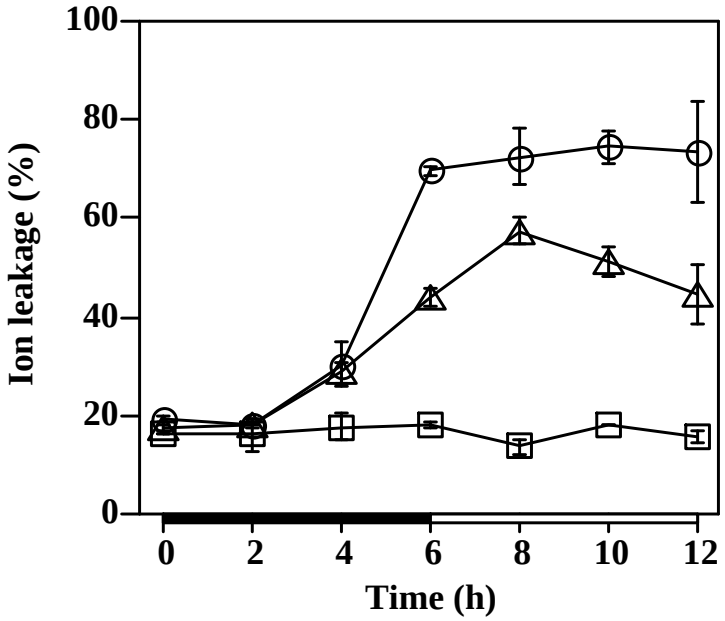

Chorismate mutase
(CM)
Chorismate
Prephenate
Arogenate
Isochorismate
synthase (ICS)
Phenylalanine
Phenylalanine
ammonia-lyase（ PAL）
Isochorismate
trans- Cinnamic acid
Isochorismate
pyruvate lyase
Benzoic acid
Benzoic acid 2-hydroxylase
(BA2H)
Salicylic acid (SA)

Figure 2. Ion leakage from, and SA level of, tobacco leaves during O_3 exposure.

(a) Ion leakage in the first through third leaves of tobacco. Tobacco was exposed to O_3 for 6 h and was then transferred to a free-air room. ○, third leaves; △, second leaves; □, first leaves.

(b) SA levels in tobacco exposed to 0.2 ppm O_3 for 6 h. ●, O_3 condition; □, control. Solid horizontal axis indicates the period of O_3 fumigation. Vertical bars represent standard deviations obtained from three replicates.

(a)


100
80
60
40
20
0
Ion leakage (%)
0
2
4
6
8
10
12
Time (h)

(b)

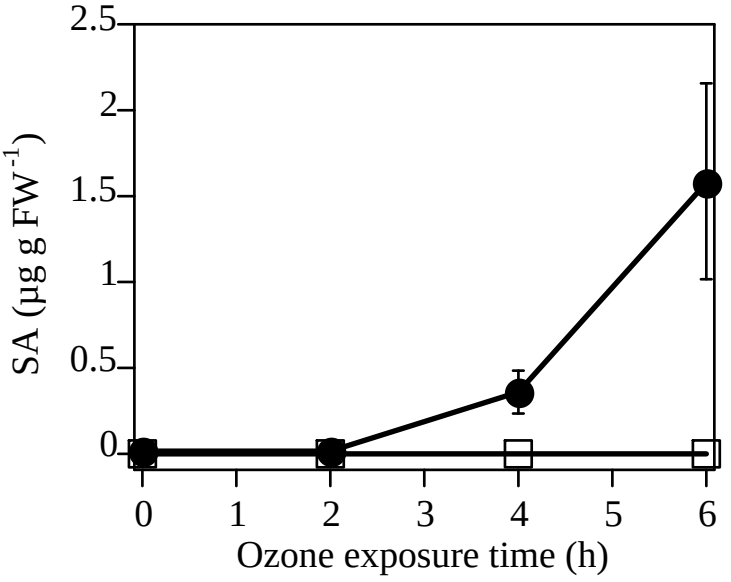

2.5
2
1.5
1
0.5
0
SA (µg g FW-1)
0
1
2
3
4
5
6
Ozone exposure time (h)

Figure 3.

(a) Clustal alignment of partial NtCM sequence in tobacco with CM in another plant. NtCM (AB182997), tobacco; LeCM (Q9STB2), *Lycopersicon esculentum.*

(b) Clustal alignment of partial NtICS sequence in tobacco with ICS in another plant. NtICS (AB182580), tobacco; CrICS (AJ006065), *Catharanthus roseus.*

(c) Southern blot analysis of *ICS* in tobacco. Twenty μg of total DNA was digested with *Dra*I, *Eco*RI, and *Hin*dIII. Hybridization was performed with isolated cDNA as a probe.

(a)

```
NtCM   1 LQSKVGRYLS SEENPFFPDK LPASIIPPSK CTPVLHPAAE CVNVNEKILD VYKKQLLPLF 60
         *********. .********. *..**** .* ********** .********* .* .*.****
LeCM  67 LQSKVGRYLA PEENPFFPDN LSDSIIPLTK CTPVLHPAAE SVNVNEKILD IYINQMLPLF 126

NtCM  61 CTDQADDEEN FATTASCDIQ LLQALSRRIH YGKFVAKVKF RDCTDQYKPL ILAKDRDALM 120
         **. ..*..* *****.**** ********** ******.*** **..*.***. ***.******
LeCM 127 CTE-VNDDAN FATTAACDIQ LLQALSRRIH YGKFVAEVKF RDSIDEYKPF ILAQDRDALM 168

NtCM 121 KLLTFEAVEE VVKKRVAKKA FVFGQQVTLN IDDNTKEAKY KVDPSLVSRL YDEWVMPLT 179
         ********** .********* ****.*.*   .**..*.*  *.** ***** *********
LeCM 169 KLLTFEAVEE MVKKRVAKKA KVFGQEVSL- -NDNAEEVKG KIDPLLVSRL YDEWVMPLT 227
```

(b)

```
NtICS   1 AYQFCLQPPQ SAAFIGNTPE QLFHRDSLSI CSEALAGTRA RGGSELLDLK IGQDLLSSAK  60
          *********. *.******** ******.*** ********** ********.* *.********
CrICS 362 AYQFCLQPPH SPAFIGNTPE QLFHRDRLSI CSEALAGTRA RGGSELLDVK IEQDLLSSAK 421

NtICS  61 DHNEFAIVRE CIRRKLEAVC SSVLIEPKKA IRKFPRVQHL YARLRGRLQT EDEEFKILSS 120
          ********** ****.***** ********** ****.***** *********. **.*******
CrICS 422 DHNEFAIVRE CIRRRLEAVC SSVLIEPKKA IRKFSRVQHL YARLRGRLQA EDDEFKILSS 481

NtICS 121 IHPTPAVCGY PTEDARAFIS ETEMFDRRMY A 151
          .********* ********** ******* ** *
CrICS 482 VHPTPAVCGY PTEDARAFIS ETEMFDRGMY A 512
```

(c)

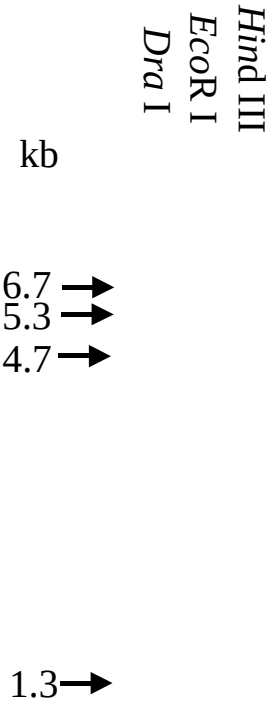


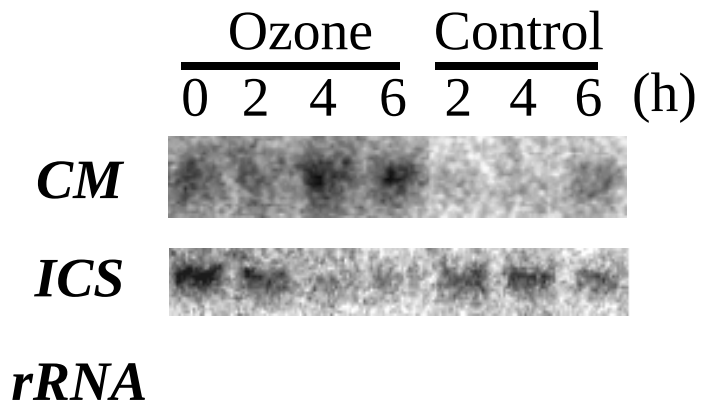
Figure 4. Transcript levels of *CM*, *ICS*, and *PAL*s in O_3-exposed tobacco.

(a) RNA gel blot analysis of *ICS* and *CM* in tobacco. Total RNAs were obtained from the first through third leaves of wild-type tobacco with or without exposure to 0.2 ppm O_3.

(b) RT-PCR analysis for *PAL A* and *PAL B*.

Numbers show the hours after the start of O_3 exposure. Columns labeled "*rRNA*" and "*Actin*" indicate equal loading of RNA.

(a)


Ozone
Control
0 2 4 6 2 4 6 (h)
CM
ICS
rRNA

(b)

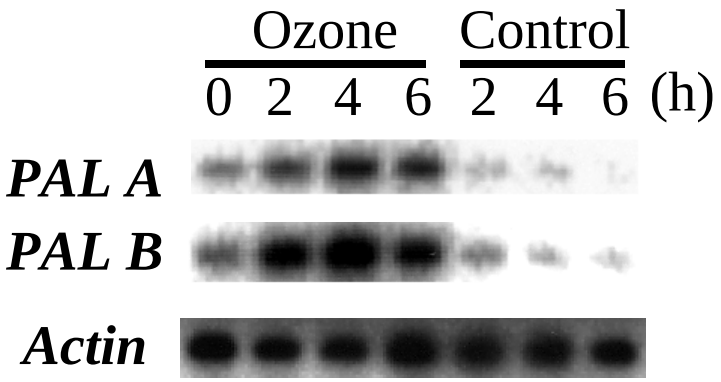

Ozone
Control
0 2 4 6 2 4 6 (h)
PAL A
PAL B
Actin

Figure 5. Incorporation of radioactivity from ^{14}C-BA into SA in O_3-exposed tobacco leaves.

Wild-type plants were exposed to 0.2 ppm O_3 for 4 h and then treated with ^{14}C-labeled BA. The amount of SA in the methanol extracts of leaves 2 h after the application of ^{14}C-labeled BA was analyzed by HPLC. Under these conditions, 3.8% (±1.3%) of the applied BA was metabolized to SA in O_3-exposed plants and 0.9% (±0.4%) in the control plants. Asterisk indicates a significant difference ($P < 0.05$; Student's t test) from the corresponding control data set. Vertical bars represent standard deviations obtained from four (ambient air) or five (O_3) replicates.

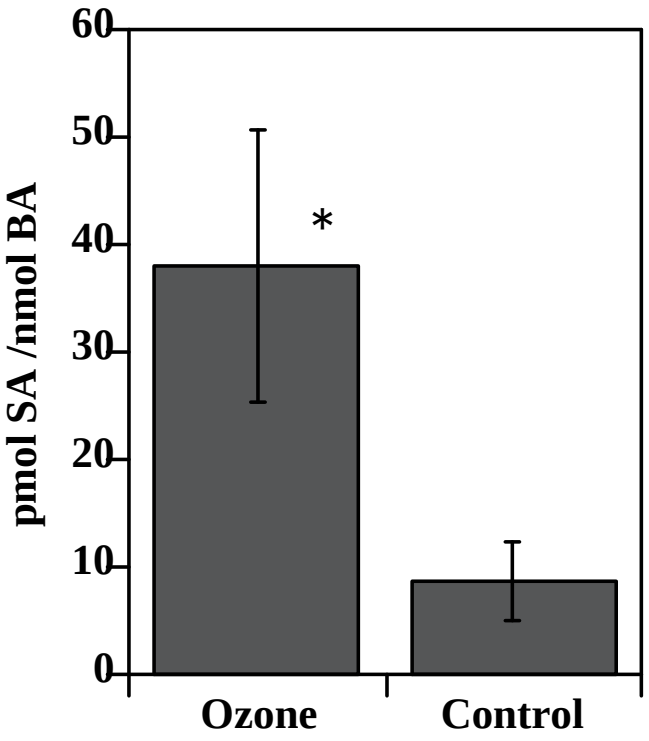

60
50
40
30
20
10
0
pmol SA /nmol BA
*
Ozone
Control

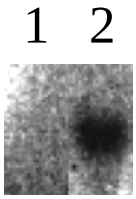
Figure 6. Ethylene emission by transgenic (As-line4) and wild-type tobacco plants during O_3 exposure. (a) Expression of anti-sense DNA. line 1, wild-type; line 2, As-line4.

(b) Ethylene production in these plants. I exposed 4- or 5-week-old plants to 0.2 ppm O_3 for 6 h. ●, wild-type; □, As-line4.

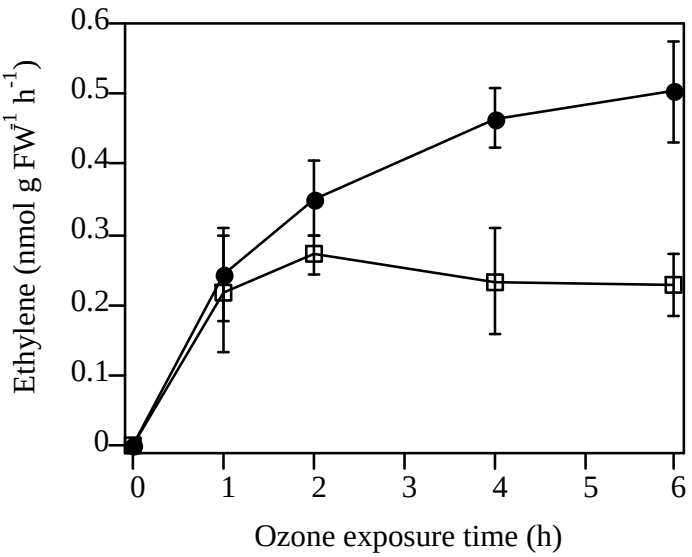
(c) Levels of ACC 4 h after the start of O_3 exposure in wild-type and As-line4 plants. White and gray bars show wild-type and As-line4 plants, respectively. Asterisk indicates significant difference ($P < 0.05$; Student's *t* test) from the corresponding data set for wild-type plants.

Vertical bars represent standard deviations obtained from three replicates.

(a)


1 2

(b)


Ethylene (nmol g FW-1 h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6
Ozone exposure time (h)

(c)

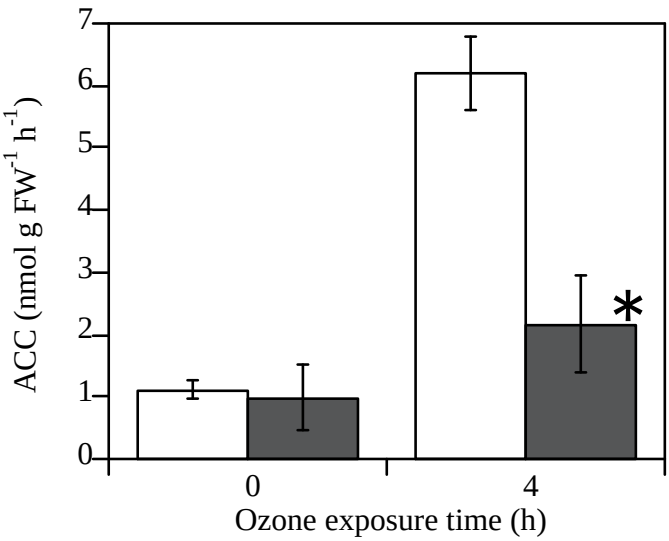

ACC (nmol g FW-1 h-1)
7
6
5
4
3
2
1
0
*
0 4
Ozone exposure time (h)

Figure 7. Damage in wild-type and As-line4 tobacco leaves exposed to 0.2 ppm O_3.

Wild-type and As-line4 tobacco leaves were exposed to 0.2 ppm O_3 for 6 h and then cultivated in O_3-free air for 18 h.

(a) Photo-image of O_3-induced damage. Numbers indicate the first, second, and third leaves.

(b) Extent of visible injury on the leaves of transgenic (As-line4) and wild-type tobacco after O_3 exposure. White and gray bars show wild-type and As-line4 plants, respectively. Areas of leaf injury on the first, second, and third leaves were measured with an image analyzer. White bar: wild-type; gray bar: As-line4. Asterisks indicate significant difference ($P < 0.05$; Student's *t* test) from the corresponding data set of wild-type plants. Vertical bars represent standard deviations obtained from 10 replicates.

(c) Ion leakage from wild-type and As-line4 tobacco leaves exposed to O_3 for 6 h, then left in ambient air for 6 h. The extent of ion leakage was measured at various times after the onset of O_3 fumigation. Solid horizontal axis indicates the period of O_3 fumigation. Vertical bars represent standard deviations obtained from six replicates. ○, third leaves; △, second leaves; □, first leaves; solid line, wild-type; dotted line, As-line4.

(a)

1

3

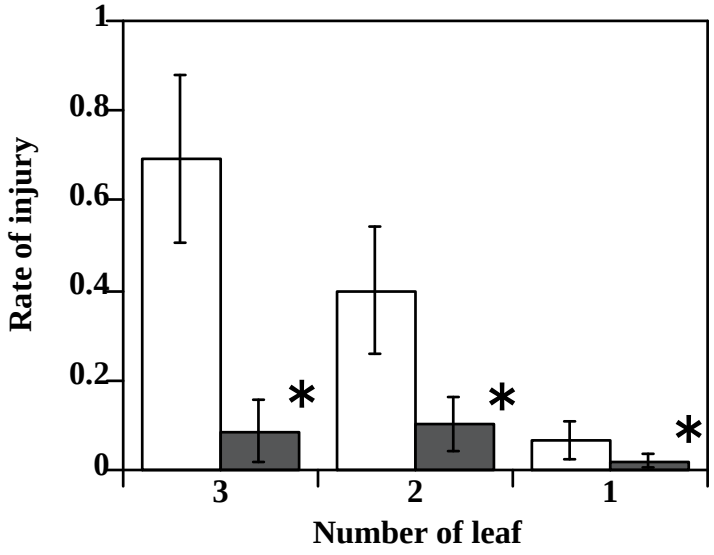
3

2

2

Wild-type As-line4

(b)



(c)

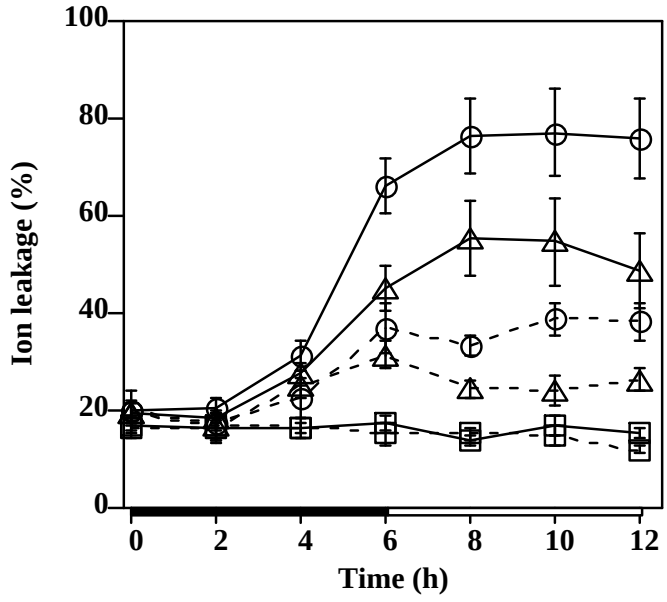


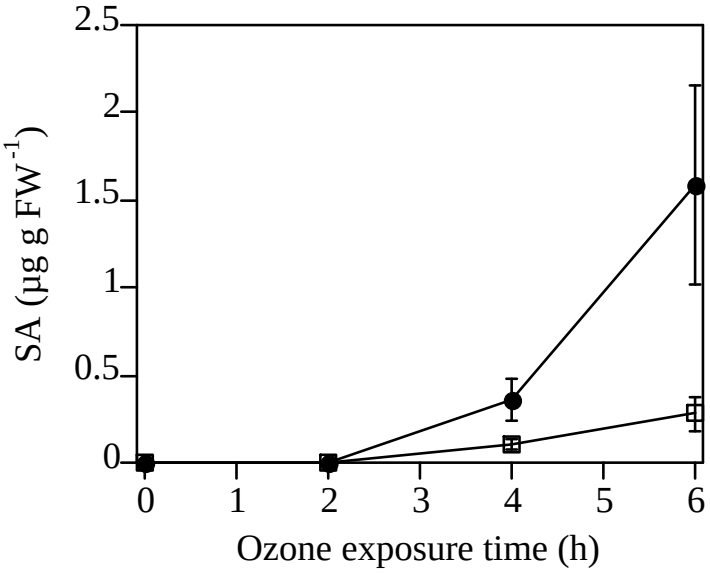
Figure 8. SA levels and PAL activity in wild-type and As-line4 tobacco leaves exposed to 0.2 ppm O_3.

(a) SA levels of the first through third leaves of wild-type and As-line4 tobacco exposed to 0.2 ppm O_3 for 6 h.

(b) PAL activity of the first through third leaves of wild-type and transgenic (As-line4) tobacco exposed to 0.2 ppm O_3 for 6 h.

Solid horizontal axis indicates the period of O_3 fumigation. ●, wild-type; □, As-line4. Vertical bars represent standard deviations obtained from three replicates.

(a)


SA (µg g FW-1)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
Ozone exposure time (h)

(b)

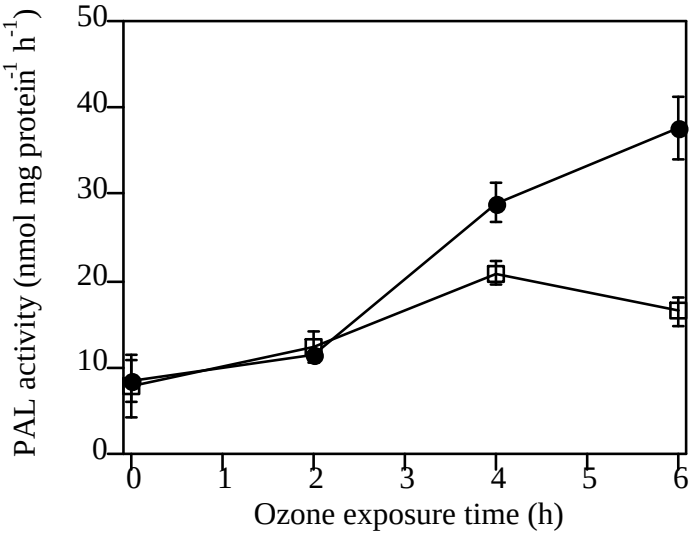

PAL activity (nmol mg protein-1 h-1)
50
40
30
20
10
0
0
1
2
3
4
5
6
Ozone exposure time (h)

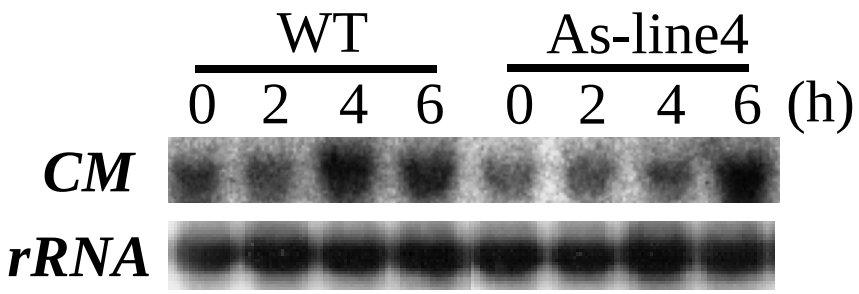
Figure 9. Transcript levels of *CM* and *PAL*s in O_3-exposed tobacco plants.

(a) RNA gel blot analysis for *CM* in wild-type tobacco and As-line4.

(b) RT-PCR analysis for *PAL A* and *PAL B*.Total RNAs were obtained from the first through third leaves from each plant.

Numbers show hours after the start of O_3 exposure. Columns labeled "*rRNA*" and "*Actin*" indicate equal loading of RNA.

(a)


WT
As-line4
0 2 4 6 0 2 4 6 (h)
CM
rRNA

(b)

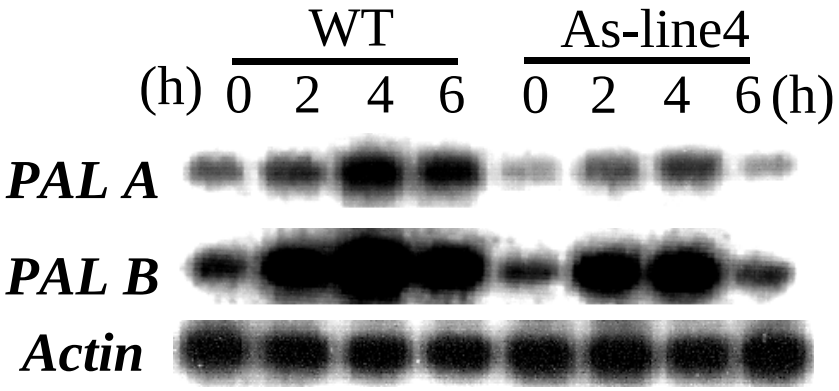

WT
As-line4
(h) 0 2 4 6 0 2 4 6 (h)
PAL A
PAL B
Actin

Figure 10. Damage in the leaves of Bel W3 and Bel B exposed to 0.2 ppm O_3.

(a) Photo-image of O_3-induced damage.

(b) Ethylene emission by Bel W3 and Bel B during O_3 exposure.

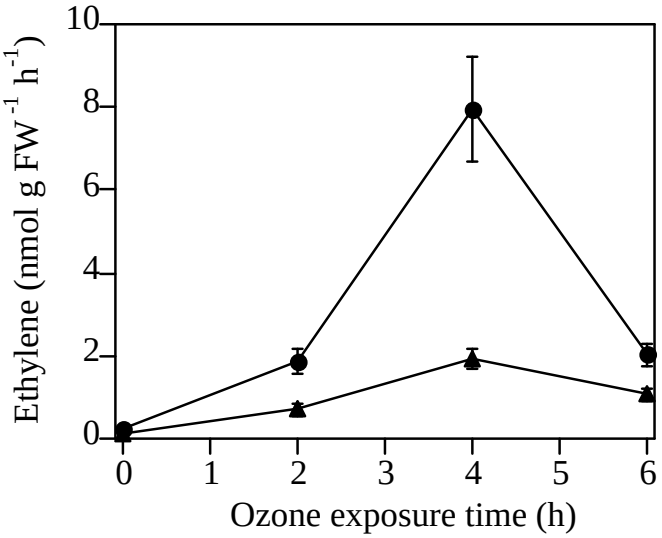
(c) SA levels in Bel B and Bel W3. ●, Bel W3; ▲, Bel B.

Four or five-week-old Bel W3 and Bel B plants were exposed to 0.2 ppm O_3 for 6 h and then kept in O_3-free air for 18 h. Vertical bars represent standard deviations obtained from three replicates.

(a)

Bel B Bel W3

(b)



(c)

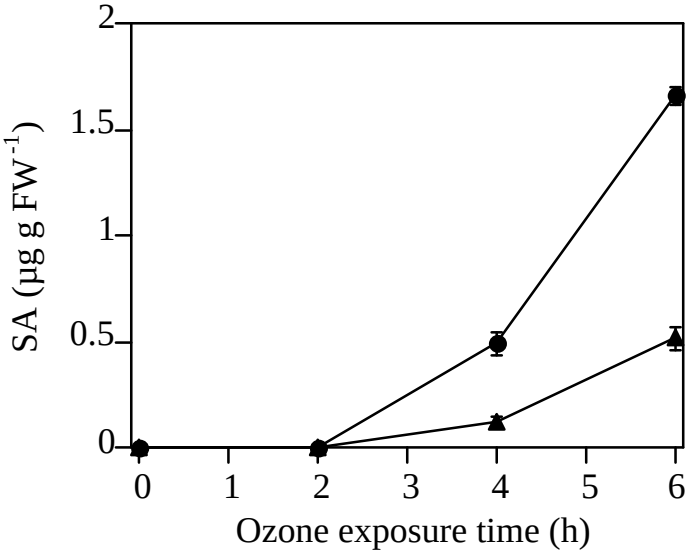


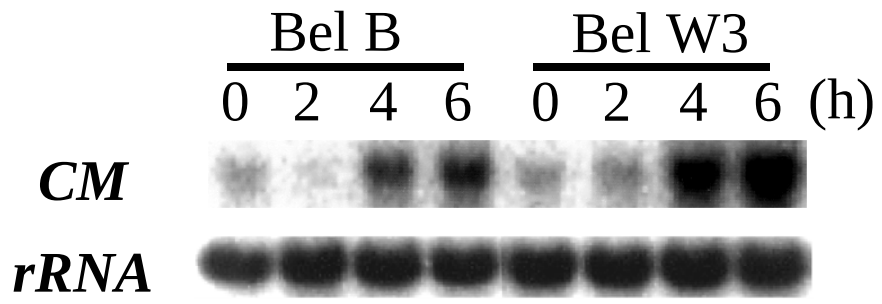
Figure 11. Transcript levels of *CM* and *PAL*s in O_3-exposed tobacco plants.

(a) RNA gel blot analysis for *CM* in Bel B and Bel W3.

(b) RT-PCR analysis for *PAL A* and *PAL B*.

Total RNAs were obtained from the first through third leaves from each plant. Numbers show hours after the start of O_3 exposure. Columns labeled "*rRNA*" and "*Actin*" indicate equal loading of RNA.

(a)


Bel B
Bel W3
0 2 4 6 0 2 4 6 (h)
CM
rRNA

(b)

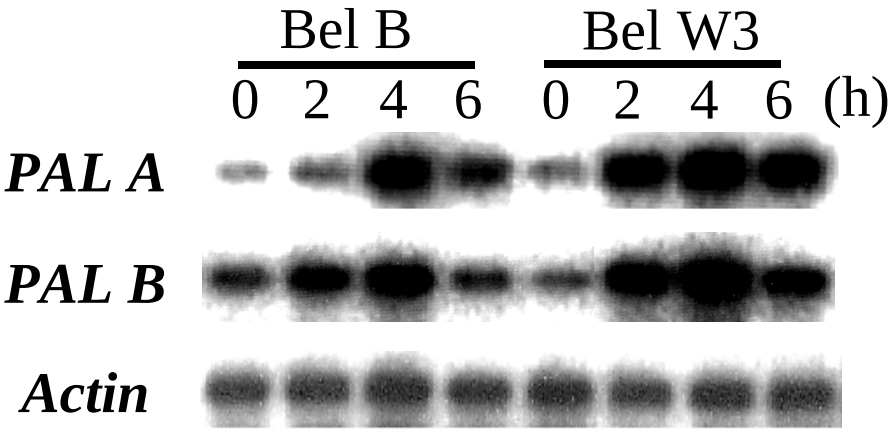

Bel B
Bel W3
0 2 4 6 0 2 4 6 (h)
PAL A
PAL B
Actin

Figure 12.

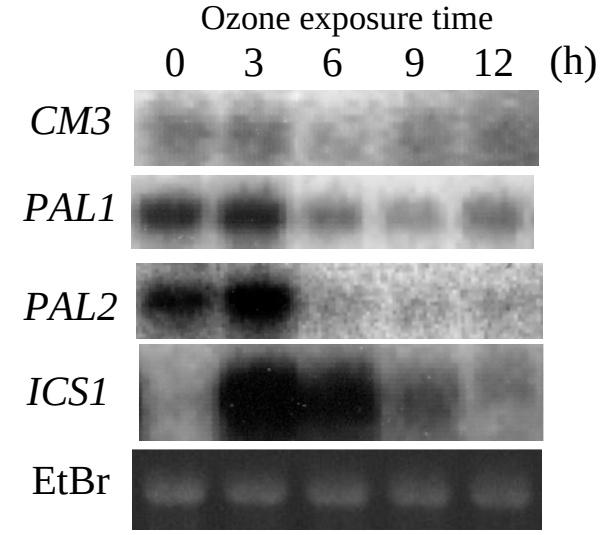
(a) RNA gel blot analysis of *ICS1*, *PAL1*, *PAL*2 in O_3-exposed *Arabidopsis*. Numbers show the hours after the start of O_3 exposure. Columns labeled “EtBr” indicate equal loading of RNA.

(b) ICS activity of wild-type *Arabidopsis* and *ics1*. ●, wild-type; □, *ics1*.

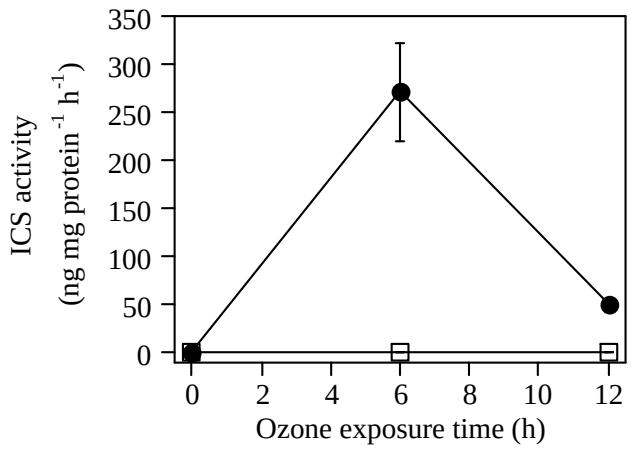
(c) SA levels in wild-type *Arabidopsis* and *ics1*. ●, wild-type; □, *ics1*. Vertical bars represent standard deviations obtained from three replicates.

(d) RNA gel blot analysis of *PR-1* in O_3-exposed *Arabidopsis*. Numbers show the hours after the start of O_3 exposure. Columns labeled “*rRNA*” indicate equal loading of RNA. Two-week-old plants were exposed to 0.2 ppm O_3 for 12 h. Vertical bars represent standard deviations obtained from three replicates.

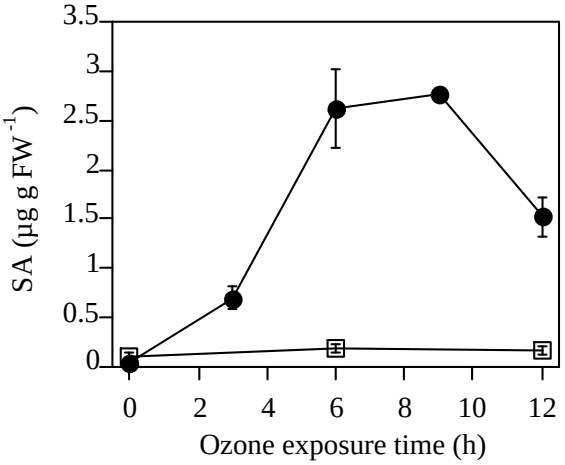
(a)


Ozone exposure time
0 3 6 9 12 (h)
CM3
PAL1
PAL2
ICS1
EtBr

(b)


ICS activity
(ng mg protein-1 h-1)
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12
Ozone exposure time (h)

(c)


SA (µg g FW-1)
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12
Ozone exposure time (h)

(d)

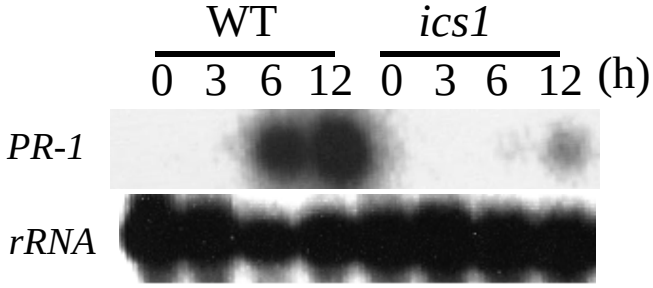

WT
ics1
0 3 6 12 0 3 6 12 (h)
PR-1
rRNA

Figure 13. SA levels of wild-type *Arabidopsis*, *etr1*, *ein2*, *npr1* and NahG.

Two-week-old plants were exposed to 0.2 ppm O_3 for 12 h. White bars (left side), 0 h; Hatched bars (middle), 6 h; Black bars (right side), 12h. Vertical bars represent standard deviations obtained from three replicates.

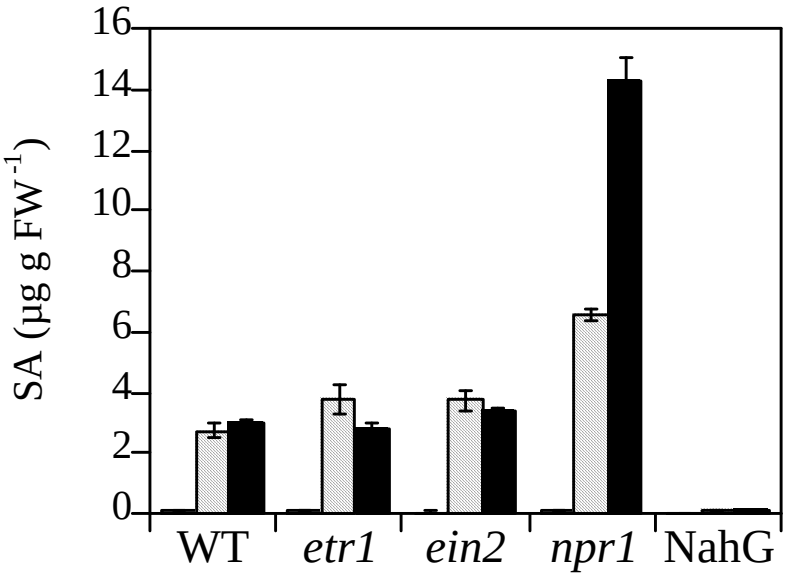

16
14
12
10
8
6
4
2
0
SA (µg g FW-1)
WT
etr1
ein2
npr1
NahG

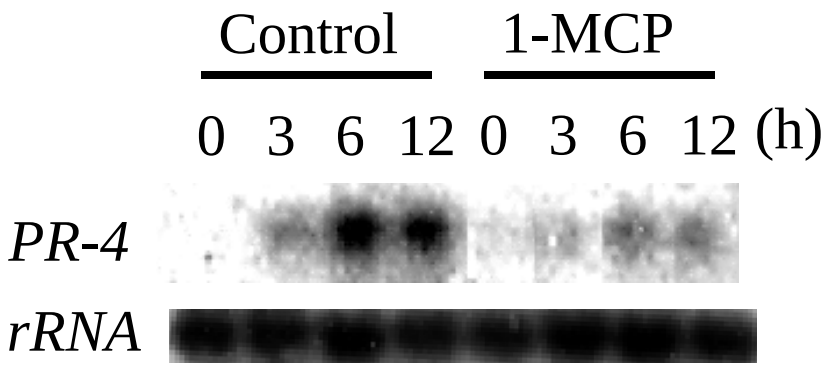
Figure 14.

(a) RNA gel blot analysis of *PR-4* in O_3-exposed *Arabidopsis* with pretreatment of 1-MCP.

(b) SA levels of O_3-exposed *Arabidopsis* with pretreatment of 1-MCP.●, control; □, 1-MCP-pretreated plants. Vertical bars represent standard deviations obtained from three replicates.

Two-week-old plants were exposed to 0.2 ppm O_3 for 12 h. Numbers show the hours after the start of O_3 exposure. Columns labeled "*rRNA*" indicate equal loading of RNA.

(a)


Control
1-MCP
0 3 6 12 0 3 6 12 (h)
PR-4
rRNA

(b)

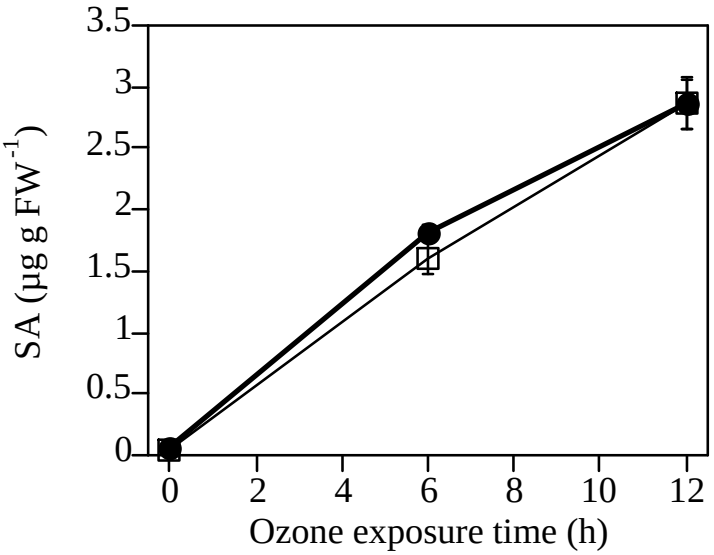

SA (μg g FW-1)
3.5
3
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12
Ozone exposure time (h)

Figure 15.

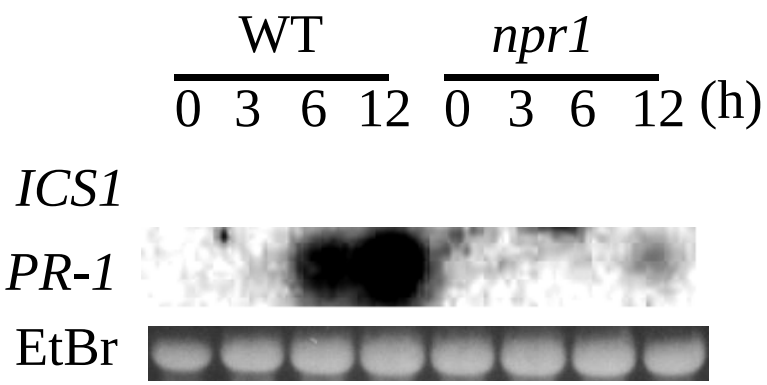
(a) RNA gel blot analysis of *ICS1* and *PR-1* in O_3-exposed wild-type *Arabidopsis* and *npr1*.

(b) RNA gel blot analysis of *ICS1* in O_3-exposed wild-type *Arabidopsis* and NahG.

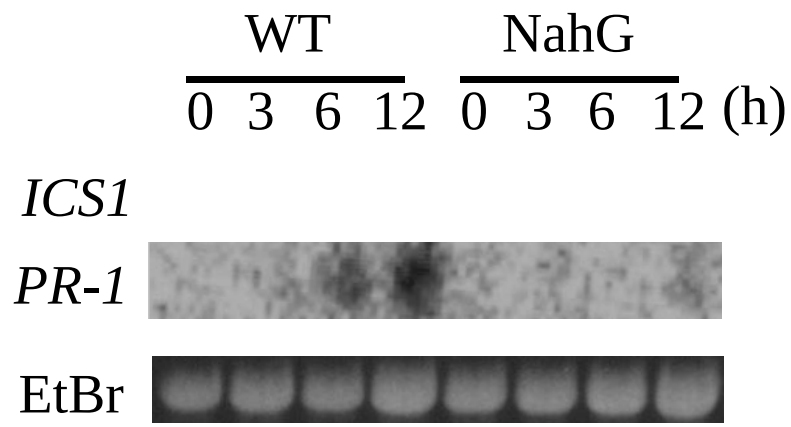
(c) ICS activity of O_3-exposed wild-type *Arabidopsis*, *npr1* and NahG. ●, wild-type; □, *npr1*; ▲, NahG. Vertical bars represent standard deviations obtained from three replicates.

Two-week-old plants were exposed to 0.2 ppm O_3 for 12 h. Numbers show the hours after the start of O_3 exposure. Columns labeled “EtBr” indicate equal loading of RNA.

(a)


WT
npr1
0 3 6 12 0 3 6 12 (h)
ICS1
PR-1
EtBr

(b)


WT
NahG
0 3 6 12 0 3 6 12 (h)
ICS1
PR-1
EtBr

(c)

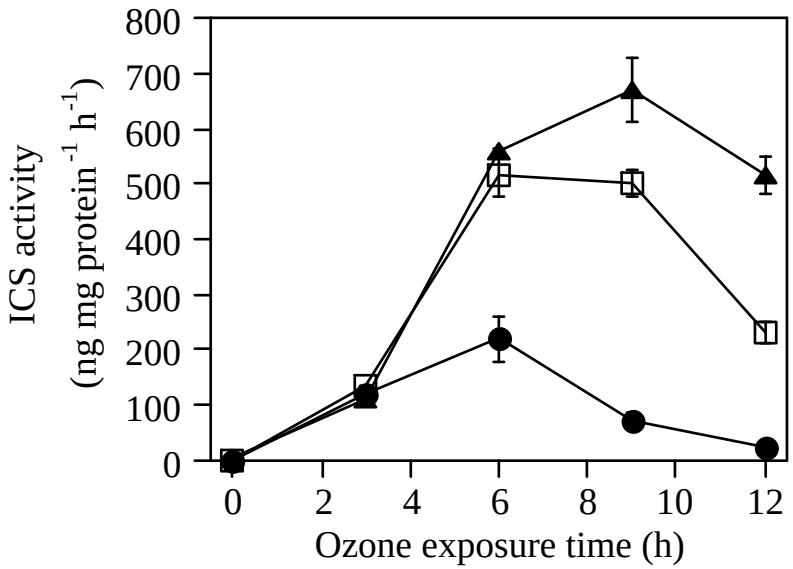

ICS activity
(ng mg protein-1 h-1)
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12
Ozone exposure time (h)

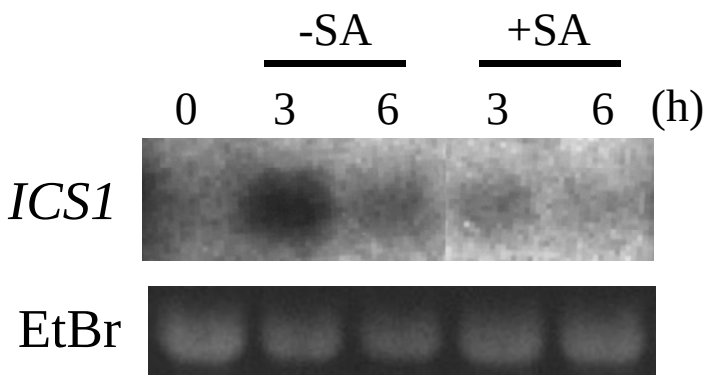
Figure 16.

(a) RNA gel blot analysis of *ICS1* in O_3-exposed *Arabidopsis* with treatment of SA. Columns labeled “EtBr” indicate equal loading of RNA.

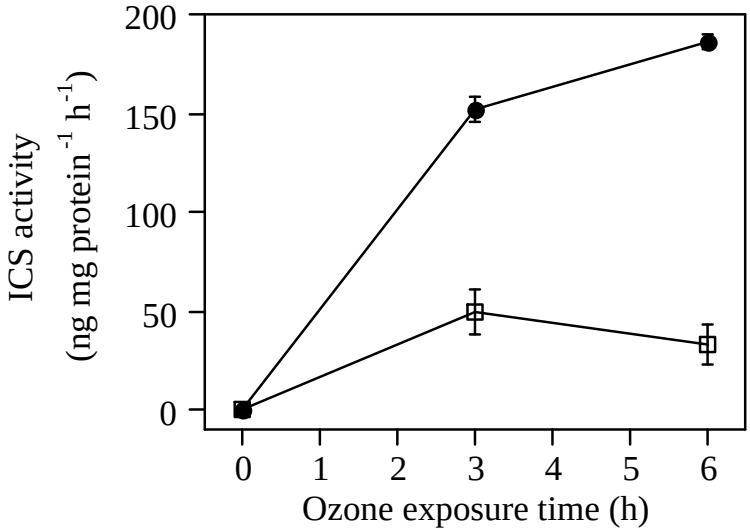
(b) ICS activity of O_3-exposed *Arabidopsis* with SA treatment. ●, control; □, SA-treated plants. Vertical bars represent standard deviations obtained from three replicates.

Two-week-old plants were exposed to 0.2 ppm O_3 for 6 h. Numbers show the hours after the start of O_3 exposure.

(a)


-SA
+SA
0
3
6
3
6
(h)
ICS1
EtBr

(b)


200
150
100
50
0
ICS activity
(ng mg protein-1 h-1)
0
1
2
3
4
5
6
Ozone exposure time (h)

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